Appendix A

ANNEX

- REPORT -

Title:
Comparison of short versus full length ALF-5755 activity on primary culture of rat hepatocytes

STUDY MANAGEMENT

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I. Aim and design of the study

The aim of this study was to investigate the biological activity of sALF-5755 (36-175 HIP/PAP form) compared to that of full-length ALF-5755 (Met-27-175 HIP/PAP form).

For this purpose we compared the biological activity of
- a standard batch of ALF-5755 containing more than 97% of Met-27-175 HIP/PAP form, with
- a batch comprising sALF-5755 containing more than 93% of 36-175 HIP/PAP obtained by treating the standard batch with Trypsin.

The biological test for studying HIP/PAP activity was the caspase 3 cleavage inhibition test on rat hepatocytes in primary culture under the stimulation of an apoptotic stress.

II. Materials and Methods

1) Test items

a. ALF-5755 batches

Experiments were performed:
- For FL-ALF-5755: batch PX7-F1-ALF-5755-291007 was manufactured and provided by PX'Pharma. This batch was received at Inserm U785 in November 2007 at a concentration of 344 ng/mL; upon arrival, it was aliquoted and stored at -80°C.
- For sALF-5755: batch I13-D150 was prepared in PX'Pharma facilities by processing 6 mL of batch PX7-F1-ALF-5755-291007 onto a trypsin column. 3 vials of 200 μL (concentration:130 ng/mL) were sent to Inserm U785 and immediately stored at -80°C.

The formulation buffer was also provided by PX'Pharma.

The table 1 below recapitulates the composition of the 2 batches.

Table 1

Batch reference	HIP/PAP form	
	36-175 HIP/PAP	Met-27-175 HIP/PAP
PX7-F1-ALF-5755-291007	3%	97%
I13-D150	93.75%	6.25%

b. ALF-5755 dosage preparation

ALF-5755 was applied as a solution in its formulation buffer added to the culture medium. The effect of increasing doses of ALF-5755 ranging from 20 to 4000 ng/mL was investigated. The dosage forms were prepared extemporaneously. ALF-5755 was mixed with the required quantity of medium in order to achieve the maximal tested concentration of 4000ng/mL. Lower concentrations were obtained by diluting the ALF-5755 4000ng/mL solution with appropriate volumes of culture medium.

2) Cell culture

Fresh rat hepatocytes were cultured following the standard operating procedure (SOP) IVTA-SOP1 (see Appendix4).

Briefly, primary hepatocytes were prepared from adult female Wistar rats (5 to 8 weeks old, Charles River Laboratories Inc. MA, USA) according to a published experimental procedure[7,8]. A standard 2-step liver perfusion was performed (500 mL of 40°C pre-warmed Hepes pH 7,65 + EGTA 0.09% followed by 300 mL Hepes / collagenase 0.25 mg/mL / $CaCl_2$ 0.75 mg/mL) to avoid massive hepatocytes death during trituration. Tissue dissociation was performed in L15 medium complemented with antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL), fungizone (250 ng/mL), and BSA (1 mg/mL). After filtration on 70 µM nylon membrane, cells were centrifuged at 600 rpm for 2 minutes to specifically pull down hepatocytes. Cells were rinsed twice in complemented L15 medium. Viable cells were counted by Trypan blue exclusion and platted in 10% FCS complemented Williams's medium + antibiotics (penicillin 100 units/mL, streptomycin 100 µg/mL), fungizone (250 ng/mL), and BSA (1 mg/mL). 30,000 cells per well in 96 well plates were seeded. Once attached (3 to 4 hours post plating) cells were switched to FCS-free complemented William's medium, which was replaced every day.

Post- trituration viability must be as high as possible since it is a marker for cell integrity, which will impact on the cell response to apoptotic stimuli and rescuing capacities of the tested drugs. Viability below 70% would disqualify the experiments. In addition, for caspase3 inhibition experiments, the number of living cells per well in the basal conditions (no treatment) at the time of apoptotic induction should be reasonably high to allow a large panel of rescuing response when treated. Caspase 3 induction should reach a magnitude of about 4 to 10 in the control condition (apoptotic condition) to maintain a certain number of living cells and allow cell death rescue. Not meeting these criteria would disqualify the experiments.

3) Reagents

All the reagents used during the course of the experiments are listed below:

Reagent	**Supplier**	**Reference**
HEPES	Sigma	H6147
Sterile water	B. Braun	0066571E
NaCl	Sigma	S5886
Sodium phosphate	Sigma	S5136
Potassium chloride	Sigma	P5405
EGTA	Sigma	E3889
CaCl2	Sigma	C7902
Collagenase	Sigma	C5138
William's medium + glutamax	Gibco Invitrogen	32551-020
BSA	PAA	K11-013
Penicillin/ Streptomycin	Gibco Invitrogen	15140-122
FCS	PAA	A15-101
Fungizon	Gibco invitrogen	15290-026
Dexamethasone	Sigma Aldrich	D4902
Insulin	Sigma Aldrich	I0516

96 well-plates	NUNC	136101
TNFα	Sigma	T7539
Actinomycine D	Sigma	A1410
Caspase inhibitor, Ac-DEVD-CHO	Promega	G5961
Caspase Glo 3/7	Promega	G8091

4) Induction of apoptosis and read-out

Apoptosis was induced in cultured hepatocytes by subjecting them to a combination of Tumor Necrosis Factor α (TNFα) and ActinomycinD at 10/50 or 20/50 ng/mL, respectively. This was based on previous work[9] and data (see reports IVTA-Rat1 to 3). Caspase 3 cleavage (caspase 3 activity) was measured with Promega Casp3-7 kit. Experiments were done as recommended by the provider. Each condition was tested in octoplicates per experiment. The effect of increasing doses of both forms of ALF-5755 ranging from 20 to 4000 ng/mL was investigated.

5) Statistical analyses

Raw data are expressed as arbitrary unit of chemoluminescence. In each experiment, data are compared to a positive control (PC: apoptotic stimulus) and a negative control (NC: no apoptotic stimulus = basal condition). Statistical evaluations of significance within experiments were applied on raw data since all measurements were done simultaneously.

However, from one experiment (or plate) to another, the quantity of chemoluminescence varies. Therefore, to perform inter-experiment (or plate) analyses, we normalized all data to their respective negative and positive controls by calculating the following ratio for each chemoluminescence value X:

R_X = (X – mean of NC) / (mean of PC – mean of NC).

By definition the mean of NC will be 0 when the mean of PC will be 1. This ratio reflects the % of caspase 3 activity when treated by ALF-5755.

A dose-response being performed for each condition, non parametric Kruscall-Wallis tests followed by Dunns post-hoc tests were applied to reveal ALF-5755 effects according to batches. IC_{50} were calculated in every condition. Two-ways ANOVA were performed to compare batches and post-hoc Bonferroni were applied to reveal effects of ALF-5755 concentration.

All indexes of fidelity were calculated for an interval of confidence of 95%. Analyses were performed with Graph-Pad software.

III. Results

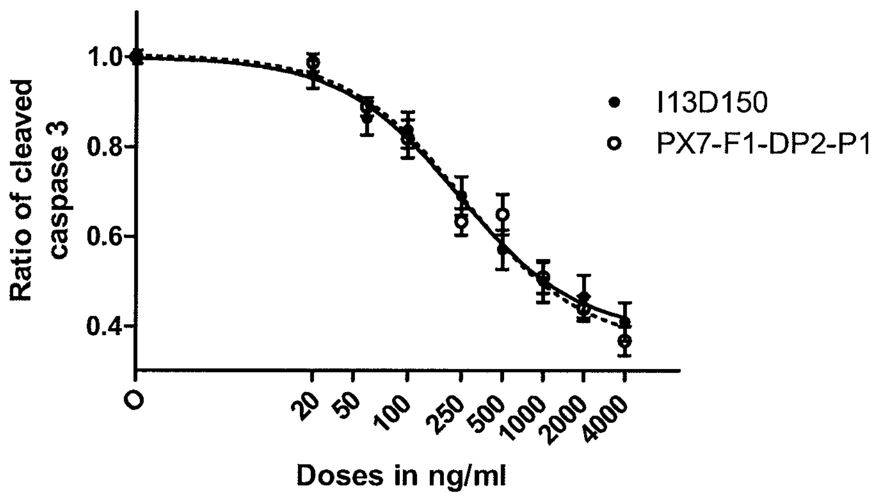
1) Assessment of sALF-5755 vs FLALF-5755 biological activity

Two experiments (080715-APOP127, 080721-APOP129) were performed with apoptosis induction mediated through TNFα/ActD combination at 10/50 and 20/50 ng/mL. For each condition 8 replicates were performed. ALF-5755 activity was comparable in the 2 conditions of TNFα for both ALF-5755 forms, therefore the corresponding data were pooled for analyses.

Calculated IC_{50} for PX7-F1-ALF-5755-291007 and I30-D150 were 264, and 238 ng/mL respectively, with overlapping intervals of confidence (175-399 and 139-410). One way

ANOVAs showed statistical significant effect of ALF-5755 from 100 ng/mL onward, for the 2 batches with P values below 0.0001 from 250ng/mL for sALF-5755 and from 100ng/mL for FL-ALF-5755. Two ways ANOVA showed no difference in activity for the two forms of ALF-5755 with $F_{(558,1)} = 0.003$, $P = 0.95$ and no interaction with $F_{(558,8)} = 0.68$, $P = 0.71$. However there was a statistically significant effect of ALF-5755 with $F_{(558,8)} = 82.3$, $P < 0.0001$. As seen in *Graph 1*, Bonferroni post-hoc tests showed no difference for each ALF-5755 form, at all concentrations with all P values above 0.05.
All data can be found in "Archive/Archive truncated" files.

Figure 1: ALF-5755 batch PX7-F1-DP2-P1 and I30-D150 display the same anti-apoptotic activity



IV. Conclusion

These results show that the activity of 36-175 HIP/PAP (sALF-5755) was as high as that of the Met-27-175 HIP/PAP (full length ALF-5755) in rat hepatocyte primary cultures. The IC_{50} of the two proteins are comparable (264, and 238 ng/mL, respectively) with widely overlapping intervals of confidence (175-399 and 139-410 ng/mL respectively).

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Eur. J. Biochem. 267, 1665–1671 (2000) © FEBS 2000

High expression of the human hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (*HIP/PAP*) gene in the mammary gland of lactating transgenic mice

Secretion into the milk and purification of the HIP/PAP lectin

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The human hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (*HIP/PAP*) gene was previously identified because of its increased expression in primary liver cancers and during the acute phase of pancreatitis. In normal tissues, HIP/PAP is expressed both in endocrine and exocrine cells of the intestine and pancreas. HIP/PAP is a lactose binding C-type lectin which acts as an adhesion molecule for rat hepatocytes.

The aim of the work was to study the HIP/PAP secretory pathway and to produce high levels of HIP/PAP in the milk of lactating transgenic mice. In view of its lactose C-type lectin properties, we have studied the consequences of the expression of HIP/PAP on mammary epithelial cells.

In homozygous mice, production reached 11.2 $mg \cdot mL^{-1}$ of milk. High levels of soluble and pure HIP/PAP (18.6 mg) were purified from 29 mL of milk. The purified protein was sequenced and the N-terminal amino acid of the mature HIP/PAP was identified as Glu27, thus localizing the site of cleavage of the signal peptide. The *HIP/PAP* transgene was only expressed in the mammary gland of lactating transgenic mice. HIP/PAP was detected by immunofluorescence in the whole gland, but labelling was heterogeneous between alveolar clusters, with strongly positive sparse cells. Using immuno electron microscopy, HIP/PAP was observed in all the compartments of the secretory pathway within the mammary epithelial cells.

We provide evidence that HIP/PAP is secreted through the Golgi pathway. However, the number of distended Golgi saccules was increased when compared to that found in wild-type mouse mammary cells. These modifications could be related to HIP/PAP C-type lectin specific properties.

Keywords: hepatocellular carcinoma; lactose C-type lectin; hepatocarcinoma-intestine-pancreas / pancreatic associated protein; transgenic mouse; mammary gland.

Hepatocarcinoma-intestine-pancreas/pancreatic-associated protein (HIP/PAP) is abundantly expressed in human primary liver cancers [1,2]. Our earlier investigations had shown that HIP/PAP was also expressed in normal subjects in the intestine (Paneth and neuro-endocrine cells), and the pancreas (acinar pancreatic cells and islets of Langerhans). HIP/PAP is rapidly overexpressed during the acute phase of pancreatitis [3]. It also acts as an adhesion molecule for rat hepatocytes and interacts with extracellular matrix proteins such as laminin-1 and fibronectin [2]. This protein contains a single carbohydrate recognition domain, linked to a putative signal peptide, and thus belongs to group VII of the C-type lectin family, according to Drickamer's classification [4]. We have previously shown that HIP/PAP is a lactose-binding protein [5].

A major problem encountered in attempting to understand the functions of HIP/PAP was twofold: the lack of information on its secretory pathway and the needs of large amounts of soluble purified protein. Indeed, published studies were based on small amounts of purified material obtained from pancreatic juice, and which could have been contaminated by other related proteins. They also relied on recombinant pre-HIP/PAP and on the carbohydrate recognition domain of the protein produced in *Escherichia coli* to generate specific antibodies [2]. However, both proteins were only recovered in small amounts, as an insoluble material requiring solubilization in urea. As an important secretion could not be obtained in humans, the physiological role of HIP/PAP secretion was studied after overexpression in transgenic mice.

The introduction of chimeric genes controlled by milk protein gene regulatory elements into the germline of mice has made it possible to obtain transgenic mice carrying foreign genes and secreting the corresponding proteins into milk. Whey acidic protein (WAP) is a milk protein whose synthesis is

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Abbreviations: GH, growth hormone; HIP/PAP, hepatocarcinoma-intestine-pancreas/pancreatic-associated protein; mWAP, mouse whey acidic protein; WAP, whey acidic protein.

(Received 26 November 1999, revised 17 January 2000, accepted 18 January 2000)

triggered by lactation. In transgenic mice carrying the chimeric rabbit WAP-human growth hormone (GH) or rabbit WAP-bovine GH genes, high levels of GH were secreted into milk during lactation [6,7].

The purpose of this study was to investigate the HIP/PAP secretory pathway and to purify large quantities of HIP/PAP in milk through the use of transgenic mouse technology. Transgenic mice carrying the *HIP/PAP* gene under the rabbit WAP gene regulatory element were generated. These transgenic mice synthesized the foreign protein in their mammary glands and secreted it into milk, after proteolytic cleavage of a signal peptide. This high level of production enabled the purification of large amounts of soluble recombinant HIP/PAP from the milk, and the determination of the N-terminal amino acid of the HIP/PAP protein. In addition, studies were performed on the morphological aspect of mammary cells expressing this protein from the lectin family and immunocytochemical localization of HIP/PAP in mammary epithelial cells.

MATERIALS AND METHODS

Materials

Mice C57Bl/6xCBA were provided by IFFA CREDO (L'Arbresle, France). Enhanced chemiluminescence (Luminol) was obtained from Amersham (Les Ulis, France); the FPLC Mono S column and ImageMaster densitometer from Pharmacia (Orsay, France); nitrocellulose (Nytran 13N) from Schleicher and Schüell (Epernon, France); Phosphor screens, STORM and IMAGEQUANT from Molecular Dynamics (Bondoufle, France). All chemicals and secondary horseradish peroxidase antibodies were provided by Sigma (L'Isle d'Abeau Chesnes, France). Polyclonal Ig against mouse α_{S1}-casein was provided by I. Barash (Institute of Animal Science, Israel). Preparation of the rabbit anti-HIP/PAP antiserum was as previously described [2].

Transgenic mice

Transgenic mice carrying the WAP/HIP construct were generated by microinjection into one-cell mouse zygotes of C57Bl/6xCBA hybrid strains [2]. They were identified by tail DNA analysis on Southern blots. Mouse DNA was digested with *Sac*I, and the generated fragments were separated on 1% agarose gels and transferred to Nytran 13N [2]. The presence of the transgene was detected using a 4.4-kb *Xho*I fragment derived from the upstream region of the rabbit WAP gene [6].

All experiments, including animal welfare and conditions for animal handling before slaughter, were conducted in accordance with French Ministry of Agriculture guidelines (dated 19 April 1988).

RNA isolation and Northern blot analysis

Total RNA was isolated from a range of tissues [6], and 20 μg of total RNA were denatured by formaldehyde and loaded on agarose gels. After gel electrophoresis, the gel was soaked in 50 mM NaOH, 1×NaCl/Cit for 10 min [8], and then rinsed in 10×NaCl/Cit, and RNA was transferred to Nytran 13N in the presence of 10×NaCl/Cit following the recommendations of the manufacturer. The membranes were examined under UV light to check for equal loading and transfer efficiency. RNAs were successively hybridized with probes corresponding to *HIP* cDNA (516 nucleotides), mouse *WAP* cDNA (541 nucleotides) [9] and mouse β-casein cDNA [10], as previously described [6]. Blots were exposed to x-RX Fujifilms or Phosphor screens, and radioactive signals were quantified using the IMAGEQUANT™.

Milk samples

Milk was collected at day 13 postparturition from anaesthetized mice previously injected with 0.05 U of oxytocin to stimulate milk letdown. Mouse milk was diluted (1/10) in 10 mM Tris/HCl pH 7.5, 100 mM $CaCl_2$, and centrifuged for 30 min at 40 000 *g*. The pellet was discarded and the supernatant was spun again under the same conditions. The supernatant or lactoserum was used immediately for the purification of HIP/PAP or kept frozen at −20 °C.

Purification of HIP/PAP protein from transgenic mouse milk

The resulting lactoserum (see above) was acidified to pH 4.6 by the addition of acetic acid (1 M) under stirring at 0 °C for 30 min. The precipitated material was removed by centrifugation at 110 000 *g* for 1 h in a Beckman 50.2 Ti rotor (Gagny, France). The supernatant was dialysed overnight at 4 °C against 1 L of 20 mM sodium acetate buffer pH 4.8, clarified by high speed centrifugation as above and filtered on a Millex 0.22 μm filter (Millipore, Guyancourt, France) before loading onto a Mono S HR 5/5 cation-exchange column previously equilibrated with 70 mM sodium acetate buffer pH 4.8. The flowthrough was discarded, and a 20-mL gradient of 0–500 mM NaCl in the working buffer was started when the absorbance returned to baseline. The column flow rate was 1 $mL{\cdot}min^{-1}$, and 1-mL fractions were collected. HIP/PAP-containing fractions were pooled, diluted in 5 vol. of 140 mM sodium acetate buffer at pH 4.0 and reapplied to the Mono S HR 5/5 column equilibrated with 140 mM sodium acetate buffer pH 4.0. The flowthrough was discarded and the column was developed with a 20-mL gradient ranging from 0 to 400 mM NaCl in the working buffer. The column flow rate was 1 $mL{\cdot}min^{-1}$ and 1-mL fractions were collected. Fractions containing HIP/PAP were pooled, diluted in 1 vol. of glycerol and stored at −20 °C.

Protein concentrations in the samples were determined using the Peterson protein assay [11]. Denaturing polyacrylamide gels in sodium dodecyl sulfate (12.5% acrylamide, SDS/PAGE) were performed according to Laemmli [12]. Coomassie blue staining gels were scanned and quantified using an IMAGEMASTER™.

Microsequencing

The N-terminal part of the purified 16-kDa HIP/PAP was sequenced by the protein microsequencing laboratory (Institut Pasteur, Paris, France) using automated Edman degradation.

Western blot analysis

Fragments of mammary glands and milk samples were analysed using Western blot analysis with mouse anti-(α_{S1}-casein) IgG (kindly given by I. Barash) diluted to 1 : 5000 [13], or pre-HIP IgG diluted to 1 : 5000, as previously described [2]. Blots were developed using enhanced chemiluminescence and autoradiography. Films were quantified by densitometry as above.

Electron microscopy

Tissue fragments were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.25, postfixed in 1% OsO_4 in the

same buffer, dehydrated and embedded in Epon. Sections were viewed using a Philips electron microscope.

Immunofluorescence labelling

Mammary fragments obtained immediately after dissection were fixed for 4 h with 2% paraformaldehyde in 0.1 M sodium cacodylate buffer. Fixed tissues were infused for 17 h in 40% sucrose in NaCl/P_i, frozen in liquid nitrogen, and then sectioned in 2-μm sections at −35 °C using a Reichert Cryocut (Leice; Rueil Malmaison, France). The sections were sequentially incubated with 50 mM NH_4Cl in NaCl/P_i (45 min), NaCl/P_i (1 h), 1% BSA (45 min), sheep serum diluted to 1 : 4 in NaCl/P_i/1% BSA (1 h), and rabbit anti-HIP/PAP polyclonal Ig diluted to 1 : 5000, washed, incubated with FITC-conjugated sheep anti-(rabbit IgG) IgG diluted to 1 : 300 (30 min), and finally washed with NaCl/P_i/1% BSA. Sections were mounted on a drop of Mowiol and observed with a Polyvar Reichert microscope equipped with a filter set to fluorescence.

Immunogold electron microscopy

Mammary fragments were fixed and embedded as previously described [7]. The sections were collected and immunolabelled by sequential incubations with rabbit anti-HIP/PAP polyclonal Ig diluted to 1 : 100 in NaCl/P_i/0.2% gelatin, and the secondary antibody [15 nm gold-conjugated anti-(rabbit IgG) IgG], 30 min, 1 : 300 in NaCl/P_i/0.2% gelatin.

RESULTS

Generation and characterization of WAP/HIP transgenic mouse lines

Seventeen transgenic founder mice carrying the *WAP/HIP* transgene (Fig. 1) were generated. Out of those mice, five did not transmit the transgene to their offspring, and 12 transgenic lines were developed from three males and nine females. The transgene copy number varied from one to 120, and seven lines had less than six copies. These lines were propagated for eight generations.

Purification and characterization of HIP/PAP secreted in the milk of the transgenic mice

Milk (29 mL) was collected from 30 hemizygous or homozygous transgenic mice (lines: 9, 101, 130) and used for the purification of HIP/PAP using a three-step procedure. A representative experiment is shown in Fig. 2. First, the three caseins (25, 30 and 42 kDa) were excluded by precipitation at

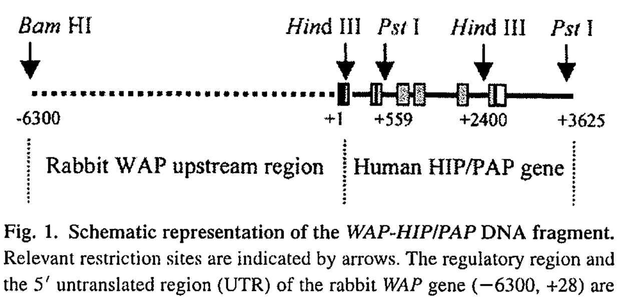

Fig. 1. Schematic representation of the *WAP-HIP/PAP* DNA fragment. Relevant restriction sites are indicated by arrows. The regulatory region and the 5′ untranslated region (UTR) of the rabbit *WAP* gene (−6300, +28) are indicated by a dotted line and black boxes, respectively. The 5′-UTR and 3′-UTR of the *HIP* gene are indicated by open boxes, the introns and 3′ flanking region of the *HIP/PAP* gene by a heavy line and the translated regions of the gene by shaded boxes.

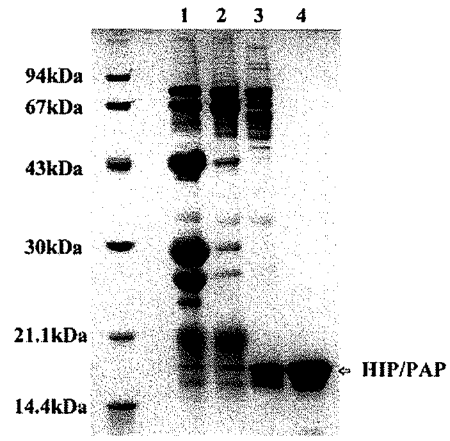

Fig. 2. Purification steps for HIP/PAP from milk. After each step, proteins were analysed using SDS/PAGE gel and stained with Coomassie blue: total milk (lane 1), supernatant obtained after casein precipitation at pH 4.6 (lane 2), fraction after removal of WAP and residual caseins using Mono S chromatography at pH 4.8 (lane 3), purified 16-kDa HIP/PAP obtained from the previous fraction after the removal of high molecular mass proteins using Mono S chromatography at pH 4.0 (lane 4).

pH 4.6 (Fig. 2, lane 2). Second, WAP (around 20 kDa) was removed, together with most of the remaining caseins using Mono S chromatography at pH 4.8 (Fig. 2, lane 3). Third, Mono S chromatography at pH 4.0 eliminated high molecular mass proteins, and pure HIP/PAP was obtained (Fig. 2, lane 4). The purification factor was about 700-fold. HIP/PAP recovery reached 18.6 mg for the entire experiment.

The HIP/PAP obtained after purification had an apparent molecular mass of 16 kDa. This molecular mass corresponded to that of HIP/PAP detected in human tissue [2]. The N-terminal part of the purified protein was sequenced and allowed us to identify the six amino acids which were: Glu, Glu, Pro, Gln, Arg and Glu. According to the *HIP/PAP* cDNA sequence, this result showed that the native HIP/PAP was cleaved between Gly26 and Glu27 eliminating a 26-amino-acid signal peptide.

Quantification of HIP/PAP protein in the milk of transgenic mice

Purified HIP/PAP was used as a standard to quantify HIP/PAP production in milk from the different transgenic lines using Western blotting (Fig. 3). No immunoreactive band was detected in nontransgenic mouse milk. Production was estimated at 0.45, 1.9, 2.3 and 4.5 mg·mL^{-1} for heterozygous lines 163, 130, 9 and 101, respectively. Levels of HIP/PAP production were not correlated to the number of transgene copies (data not shown). In one of the highest expressing lines (line 9), HIP/PAP levels reached 11.2 mg·mL^{-1} in mice homozygous for the *HIP/PAP* transgene. Thus HIP/PAP was abundantly expressed in milk.

Temporal regulation and tissue specificity of *HIP/PAP* transgene expression in the mammary gland of transgenic mice

HIP/PAP mRNAs were detected in the mammary glands of lactating transgenic mice (Fig. 4, upper panel). The level of

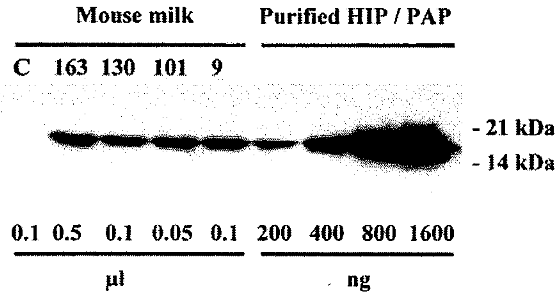

Fig. 3. HIP/PAP production in the milk of lactating transgenic mice. Protein levels were quantified by Western blotting. Milk from control, and transgenic lines 163 (0.5 µL), 130 (0.1 µL), 101 (0.05 µL), 9 (0.1 µL), and purified HIP/PAP (200, 400, 800 and 1600 ng) were loaded from left to right, respectively.

HIP/PAP mRNA varied from 1 to 10 in the different lines (Fig. 4, upper panel), and also varied within one line from one animal to another (data not shown). The mean value for *HIP/PAP* transgene expression was estimated relative to the expression of mouse *WAP* (*mWAP*) mRNA levels (Fig. 4, middle panel). For this purpose, *HIP/PAP* and *mWAP* cDNA probes of similar length were labelled to a similar specific activity and used to hybridize Northern blottings. The resulting signals were quantified using the Storm™. HIP/PAP signals were 1/18th to 1/50th of those obtained with the *mWAP* probe. *mWAP* gene expression appeared not to be affected by transgene expression (Fig. 4, middle panel). Even though the transgene was driven by upstream regions of the rabbit *WAP* gene (homologous to *mWAP* upstream regions), there was no clear competition between rabbit and *mWAP* regulatory elements.

HIP/PAP transgene expression was evaluated during the second half of pregnancy. *HIP/PAP* mRNA was detected in the mammary gland of only a small number of transgenic animals, at day 18 of pregnancy (Fig. 5, upper panel). No signals were detected at earlier stages of pregnancy or in cyclic mice (data not shown). The induction of transgene expression was thus similar to endogenous *mWAP* gene expression (Fig. 5, middle panel), and clearly began later than β-casein expression (Fig. 5, lower panel).

HIP/PAP mRNA was not detected either in the liver of the transgenic mice (Fig. 4, lanes 5 and 8), or in other tissues (data not shown).

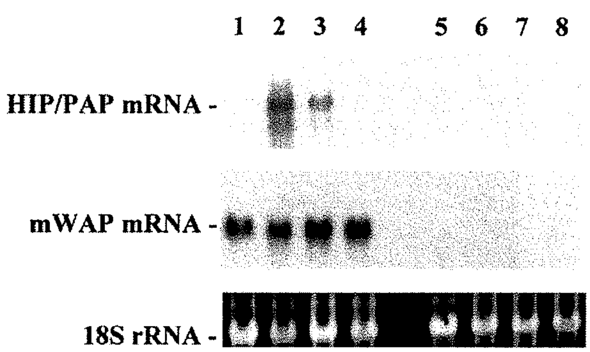

Fig. 4. Expression of the *HIP/PAP* transgene and *mWAP* gene in the mammary gland and liver of lactating transgenic mice. Total RNA (20 µg) from the mammary gland of lactating mice (lanes 1–4), and liver (lanes 5–8) were analysed using Northern blotting. Control mouse (lanes 1 and 5); transgenic line 9 (lanes 2 and 6), transgenic line 130 (lanes 3 and 7), and transgenic line 163 (lanes 4 and 8). Membranes were successively hybridized with a *HIP/PAP* cDNA probe (upper panel) and then with a *mWAP* cDNA probe (middle panel). 18S RNA were visualized by ethidium bromide staining of the membrane (lower panel). Blots were exposed to Phosphor screen or X-ray films for 4 (*mWAP* probe) to 24 h (*HIP/PAP* probe).

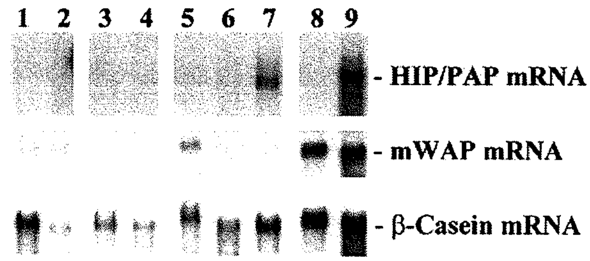

Fig. 5. Temporal expression of the *HIP/PAP* transgene in the mammary gland during lactation and pregnancy. Total RNA (20 µg), from 12-day pregnant mice (lanes 1 and 2), from 16-day pregnant mice (lanes 3–5), from 18-day pregnant mice (lanes 6 and 7) and from lactating mice (lanes 8 and 9) were analysed using Northern blotting and hybridized with a *HIP/PAP* cDNA probe (upper panel), with a *mWAP* probe (middle panel), and with a mouse β-casein probe (lower panel). Control mice (lanes 1, 3, 6, 8), transgenic line 101 (lanes 2, 5, 7, 9), and transgenic line 130 (lane 4). Blots were exposed to Phosphor screen or X-ray films for 2 h (β-casein probe), 4 h (*mWAP* probe), or 24 h (*HIP/PAP* probe).

Secretion of HIP/PAP as compared to another major milk protein, α_{S1}-casein

In order to test whether the intracellular transport and secretion of HIP/PAP occur satisfactorily in the mammary gland of transgenic mice, we compared the rates of secretion of HIP/PAP and a major protein secreted into mouse milk, α_{S1}-casein, for which an antibody was available [13]. The rate of secretion

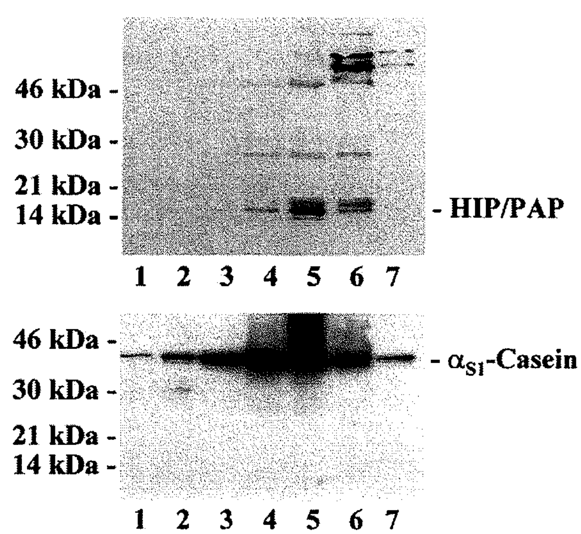

Fig. 6. Secretion of HIP/PAP into milk as compared to α_{S1}-casein. Milk (1×10^{-3}, 5×10^{-3}, 10×10^{-3}, 50×10^{-3}, 250×10^{-3} µL, lanes 1–5, respectively) and mammary gland extracts (50 and 5 µg, lanes 6 and 7, respectively) from a mouse in line 163 were loaded on the same SDS gel. The blot was incubated first with a HIP/PAP antibody (upper panel) and then with an α_{S1}-casein antibody (lower panel).

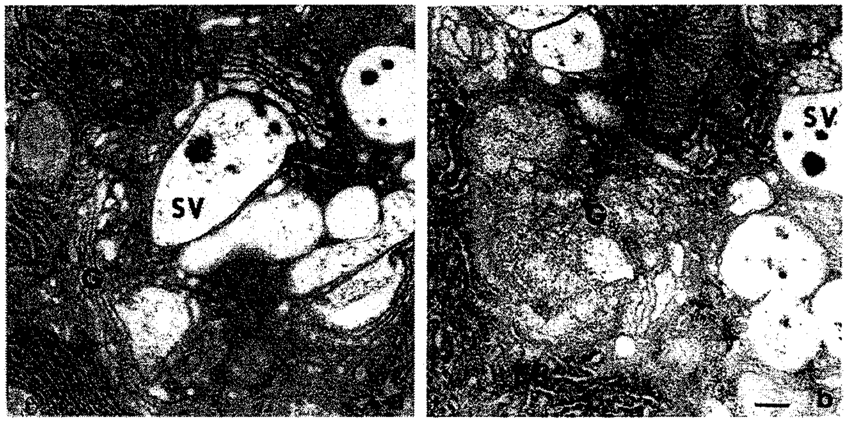

Fig. 7. Comparison of the Golgi morphology between control and transgenic mice. Control (a) and transgenic (b) mouse mammary cells at day 15 of lactation. ER, endoplasmic reticulum; G, Golgi saccules; SV, secretory vesicules. Bar, 0.5 μm.

was estimated as the amount of protein in milk relative to the amount of protein detected in mammary gland extracts from the same mouse. Amounts of proteins were estimated simultaneously by densitometric analysis of Western blotting. For HIP/PAP, the signal intensity observed for 50 μg of tissue extract was between that observed for 0.05 and 0.25 μL of milk (Fig. 6). For α_{S1}-casein, the signal observed for the same amount of tissue extract was equivalent to that observed with a much lower quantity of milk: 0.01 μL. Similar results were observed in both transgenic lines 163 and 9 (data not shown), and appeared to be independent of the level of HIP/PAP secretion. Such results tend to indicate that HIP/PAP may be less efficiently secreted into milk than α_{S1}-casein.

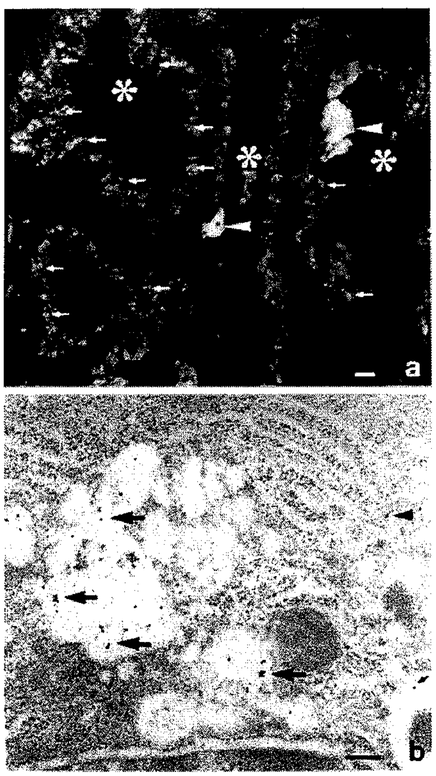

Fig. 8. Localization of HIP/PAP protein in lactating transgenic mouse mammary cells. (a) Mammary tissues were fixed and stained with HIP/PAP antibody and processed for immunofluorescence. HIP/PAP was located in the apical cytoplasm (arrows). Single cells were brightly labelled (arrowheads). A faint staining was detectable in the lumen of acini (asterisks). Bar, 10 μm. (b) Mammary tissues were processed for immunogold labelling with HIP/PAP antibody followed by a gold-conjugated secondary antibody as described in Materials and methods. Labelling was faintly detectable in the endoplasmic reticulum (arrow head), in distended Golgi saccules (arrows) and in the lumen of secretory vesicules containing casein micelles (small arrow). Bar, 0.5 μm.

Morphological aspects of lactating transgenic mouse mammary tissue

In order to ascertain whether the reduction in HIP/PAP secretion was due to an accumulation of the protein in the mammary cells, the ultrastructure of mammary epithelial cells from HIP/PAP lactating transgenic mice was compared to that of normal lactating mice. No major changes to the morphological aspect were observed in mammary epithelial cells from transgenic mice. However, numerous distended Golgi saccules and secretory vesicules, both containing an electron dense and filamentous material, were observed when compared to wild-type mouse mammary cells (Fig. 7).

Using immunofluorescence, HIP/PAP was detected in whole mammary tissue but the labelling was heterogeneous with some strongly positive alveolar clusters, as has been described for expression of other types of transgenes in the mammary gland [14]. In addition, the entire cytoplasm of sparse cells was totally labelled. Such brightly stained, single cells have already been described for lactoferrin expression in mice [15]. In strongly positive alveolar clusters, fluorescent signals were localized in the supranuclear region of mammary epithelial cells (Fig. 8a), and in the lumen of the acini. A similar immunofluorescence had been observed for caseins [7]. However, no HIP/PAP labelling was observed at the basal part of the acini.

Using electron immunogold microscopy, HIP/PAP was detected in the rough endoplasmic reticulum, in the distended Golgi saccules, in the secretory vesicles containing casein micelles and in the lumen of acini of the transgenic mouse mammary cells (Fig. 8b). HIP/PAP was mainly located in the soluble part of the compartment within secretory vesicules and the lumen of the acini.

DISCUSSION

The regulatory region of the rabbit *WAP* gene (−6300, + 28) is able to drive expression of the *HIP/PAP* gene in the mammary gland. The regulation of expression of the transgene is similar

to that of the endogenous mouse WAP gene, and expression is restricted to the mammary gland during lactation. These transgenic mice enabled us to show that HIP/PAP is secreted through the Golgi pathway, after elimination of an N-terminal signal. Moreover, this is the first model capable of producing soluble human HIP/PAP, and we were able to purify high levels of pure recombinant human HIP/PAP.

The crucial point during the purification procedure was the pH value (pH 4.8) which was the isoelectric point for caseins (the most abundant contaminant in milk) during their precipitation, and was also that necessary to keep HIP/PAP soluble during cation-exchange chromatography. We thus obtained 18.6 mg from 29 mL of transgenic mouse milk. Although publications have reported on the expression of proteins of pharmaceutical or medical interest in the milk of transgenic animals, only a few have described the purification of large amounts of protein C [16,17], α1-antitrypsin [18], tissue plasminogen activator [19], IGF-I [20], calcitonin [21], furin [22], GH [23] or parathyroid hormone [24].

The mammary gland appears to be suited to the study of the HIP/PAP secretion pathway, and enables monitoring to follow the maturation of HIP/PAP. We determined the N-terminal amino acid of HIP/PAP (Glu27) following proteolytic cleavage of a 26-amino-acid peptide. This sequence shows three features consistent with those of a peptide signal [25], namely: first, its length; second, the presence of a highly hydrophobic stretch (7 Leu, 3 Pro, 1 Trp); third, the presence of a small neutral side chain on the N-terminal side (Gly) of the cleavage site. Most signal peptides also contain a positively charged residue [25], even though the presence of basic amino acids have been shown not to be required [26]. No such sequence is present in the HIP/PAP signal sequence. HIP/PAP produced by the mammary gland had the same electrophoretic migration as that observed with HIP/PAP from the human intestine, pancreas [2], and serum [27]. These results therefore suggest that HIP/PAP is processed and secreted similarly in the transgenic mammary gland and in human tissues. Cleavage of the Gly-Glu bond of the human HIP/PAP has not been reported before, but it is interesting to note that a similar processing has been described for the rat PAPI, which is homologous to human HIP/PAP [28]: a glutamic acid in position 27 of the rat preprotein was determined by microsequencing the mature rat PAPI purified from pancreatic juice. A secretion pathway through the Golgi apparatus, after cleavage of a hydrophobic peptide, is described for most secreted proteins, including extracellular C-type lectins, in contrast with that of galectins. This other class of lectins whose secretion pathway is still unknown lacks, however, a signal sequence [29].

In order to determine whether the synthesis and intracellular transport of HIP/PAP had any apparent consequences on lactating mammary epithelial cells, the morphology of the latter was observed using electron microscopy. Golgi saccules were distended and filled with a filamentous, electron-dense material. These modifications were detected in neither control animals nor in other transgenic mice expressing different proteins such as GH or α1-antitrypsin [7,30]. The secretory pathway seemed not to be impaired, except for a slight reduction in secretion when compared with that of α_{S1}-casein. This slight perturbation could be due to the fact that high levels of lactose are synthesized in the Golgi saccules [31] and secreted into milk, and that HIP/PAP is a C-type lectin exhibiting a lactose binding activity [5]. HIP/PAP may bind to lactose-derived glycoproteins, and this may account for the morphological modifications observed in mammary epithelial cells. We previously tested HIP/PAP activity *in vitro* and we showed that HIP/PAP induced the adhesion of rat hepatocytes, and bound strongly to extracellular matrix proteins (laminin-1, fibronectin) [2]. In the mammary cells of transgenic mice, however, HIP/PAP was mainly detected in the lumen of the acini or at the apical pole and not on the basal part of the cell. Such an adhesive role for HIP/PAP is therefore unlikely in these cells. The expression of HIP/PAP in the mammary gland and its secretion into milk did not affect the health of the mother. Nor did it affect the growth of pups, suggesting that the global milk composition was similar in transgenic and control mice. This overall similarity was also observed at the protein level, as indicated by the patterns of proteins stained using Coomassie blue (data not shown).

In conclusion, a high level of soluble, human HIP/PAP was secreted in the milk of transgenic mice, indicating that the rabbit *WAP* promoter is perfectly capable of driving highly differing heterologous genes [6,30], and that HIP/PAP is properly matured during its transport through the mammary epithelial cells. The availability of large amounts of protein has made it possible to crystallize HIP/PAP, which is the first recombinant protein cristallized after production in the transgenic-mouse milk [32]. Its three-dimensional structure will now be established and should provide information concerning the function of this protein.

ACKNOWLEDGEMENTS

This work was supported by: Institut National de la Santé et de la Recherche Médicale, Association pour la Recherche contre le Cancer, Ligue Nationale contre le Cancer and Fondation de France. The authors wish to thank Mrs V. Hawken for linguistic corrections of the manuscript.

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Guidance for Industry

Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

August 1999
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Appendix C

Guidance for Industry

Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

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U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

August 1999
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TABLE OF CONTENTS

GUIDANCE FOR INDUSTRY[1]

Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

I. OBJECTIVES OF THIS DOCUMENT (1.0)[2]

A. Objective (1.1)

This guidance document provides guidance on general principles for the setting and justification, to the extent possible, of a uniform set of international specifications for biotechnological and biological products to support new marketing applications.

B. Background (1.2)

A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product, or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. *Conformance to specification* means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.

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[1] This guidance was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, March 10, 1999. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan, and the United States. This guidance was published in the *Federal Register* on August 18, 1999 (62 FR 44928), and is applicable to drug and biological products.

[2] Arabic numbers reflect the organizational breakdown in the document endorsed by the ICH Steering Committee at Step 4 of the ICH process, March 10, 1999.

Specifications are one part of a total control strategy designed to ensure product quality and consistency. Other parts of this strategy include thorough product characterization during development, upon which many of the specifications are based, adherence to good manufacturing practices, a validated manufacturing process, raw materials testing, in-process testing, stability testing, etc.

Specifications are chosen to confirm the quality of the drug substance and drug product rather than to establish full characterization and should focus on those molecular and biological characteristics found to be useful in ensuring the safety and efficacy of the product.

C. Scope (1.3)

The principles adopted and explained in this document apply to proteins and polypeptides, their derivatives, and products of which they are components (e.g., conjugates). These proteins and polypeptides are produced from recombinant or nonrecombinant cell-culture expression systems and can be highly purified and characterized using an appropriate set of analytical procedures.

The principles outlined in this document may also apply to other product types, such as proteins and polypeptides isolated from tissues and body fluids. To determine applicability, manufacturers should consult with the appropriate regulatory authorities.

This document does not cover antibiotics, synthetic peptides and polypeptides, heparins, vitamins, cell metabolites, deoxyribonucleic acid (DNA) products, allergenic extracts, conventional vaccines, cells, whole blood, and cellular blood components. A separate ICH draft guidance, *Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances,* addresses specifications and other criteria for chemical substances.

This document does not recommend specific test procedures or specific acceptance criteria, nor does it apply to the regulation of preclinical and/or clinical research material.

II. PRINCIPLES FOR CONSIDERATION IN SETTING SPECIFICATIONS (2.0)

A. Characterization (2.1)

Characterization of a biotechnological or biological product (which includes the determination of physicochemical properties, biological activity, immunochemical properties, purity, and impurities) by appropriate techniques is necessary to allow relevant specifications to be established. Acceptance criteria should be established and justified based on data obtained from lots used in preclinical and/or clinical studies, data from lots used for demonstration of manufacturing consistency, data from stability studies, and relevant development data.

Extensive characterization is performed in the development phase and, where necessary, following significant process changes. At the time of submission, the product should have been compared with an appropriate reference standard, if available. When feasible and relevant, it should be compared with its natural counterpart. Also, at the time of submission, the manufacturer should have established appropriately characterized in-house reference materials which will serve for biological and physicochemical testing of production lots. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

1. Physicochemical Properties (2.1.1)

A physicochemical characterization program will generally include a determination of the composition, physical properties, and primary structure of the desired product. In some cases, information regarding higher-order structure of the desired product (the fidelity of which is generally inferred by its biological activity) may be obtained by appropriate physicochemical methodologies.

An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic processes used by living organisms to produce them; therefore, the desired product can be a mixture of anticipated post-translationally modified forms (e.g., glycoforms). These forms may be active and their presence may have no deleterious effect on the safety and efficacy of the product (section II.A.4). The manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in preclinical and clinical studies. If a consistent pattern of product heterogeneity is demonstrated, an evaluation of the activity, efficacy, and safety (including immunogenicity) of individual forms may not be necessary.

Heterogeneity can also be produced during manufacture and/or storage of the drug substance or drug product. Since the heterogeneity of these products defines their quality, the degree and profile of this heterogeneity should be characterized to ensure lot-to-lot consistency. When these variants of the desired product have properties comparable to those of the desired product with respect to activity, efficacy, and safety, they are considered product-related substances. When process changes and degradation products result in heterogeneity patterns that differ from those observed in the material used during preclinical and clinical development, the significance of these alterations should be evaluated.

Analytical methods to elucidate physicochemical properties are listed in appendix A. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

For the purpose of lot release (section IV), an appropriate subset of these methods should be selected and justified.

2. *Biological Activity (2.1.2)*

Assessment of the biological properties constitutes an equally essential step in establishing a complete characterization profile. An important property is the biological activity that describes the specific ability or capacity of a product to achieve a defined biological effect.

A valid biological assay to measure the biological activity should be provided by the manufacturer. Examples of procedures used to measure biological activity include:

- Animal-based biological assays, which measure an organism's biological response to the product;

- Cell culture-based biological assays, which measure biochemical or physiological response at the cellular level; and

- Biochemical assays, which measure biological activities such as enzymatic reaction rates or biological responses induced by immunological interactions.

Other procedures, such as ligand and receptor binding assays, may be acceptable.

Potency (expressed in units) is the quantitative measure of biological activity based on the attribute of the product that is linked to the relevant biological properties, whereas quantity (expressed in mass) is a physicochemical measure of protein content. Mimicking the biological activity in the clinical situation is not always necessary. A correlation between the expected clinical response and the activity in the biological assay should be established in pharmacodynamic or clinical studies.

The results of biological assays should be expressed in units of activity calibrated against an international or national reference standard, when available and appropriate for the assay utilized. Where no such reference standard exists, a characterized in-house reference material should be established and assay results of production lots reported as in-house units.

Often, for complex molecules, the physicochemical information may be extensive but unable to confirm the higher-order structure which, however, can be inferred from the biological activity. In such cases, a biological assay, with wider confidence limits, may be acceptable when combined with a specific quantitative measure. Importantly, a biological assay to measure the biological activity of the product may be replaced by physicochemical tests only in those instances where:

- Sufficient physicochemical information about the drug, including higher-order structure, can be thoroughly established by such physicochemical methods, and relevant correlation to biologic activity demonstrated; and

- There exists a well-established manufacturing history.

Where physicochemical tests alone are used to quantitate the biological activity (based on appropriate correlation), results should be expressed in mass.

For the purpose of lot release (section IV), the choice of relevant quantitative assay (biological and/or physicochemical) should be justified by the manufacturer.

3. Immunochemical Properties (2.1.3)

When an antibody is the desired product, its immunological properties should be fully characterized. Binding assays of the antibody to purified antigens and defined regions of antigens should be performed, as feasible, to determine affinity, avidity and immunoreactivity (including cross-reactivity). In addition, the target molecule bearing the relevant epitope should be biochemically defined and the epitope itself defined, when feasible.

For some drug substances or drug products, the protein molecule may need to be examined using immunochemical procedures (e.g., enzyme linked immunosorbent assay (ELISA), Western-blot) utilizing antibodies that recognize different epitopes of the protein molecule. Immunochemical properties of a protein may serve to establish its identity, homogeneity, or purity, or serve to quantify it.

If immunochemical properties constitute lot release criteria, all relevant information pertaining to the antibody should be made available.

4. Purity, Impurities, and Contaminants (2.1.4)

- **Purity**

The determination of absolute, as well as relative, purity presents considerable analytical challenges, and the results are highly method dependent. Historically, the relative purity of a biological product has been expressed in terms of specific activity (units of biological activity per milligram of product), which is also highly method dependent. Consequently, the purity of the drug substance and drug product is assessed by a combination of analytical procedures.

Due to the unique biosynthetic production process and molecular characteristics of biotechnological and biological products, the drug substance can include several molecular entities or variants. When these molecular entities are derived from anticipated post-translational modification, they are part of the desired product. When variants of the desired product are formed during the manufacturing process and/or storage and have properties comparable to the

desired product, they are considered product-related substances and not impurities (section II.A.1).

Individual and/or collective acceptance criteria for product-related substances should be set, as appropriate.

For the purpose of lot release (section IV), an appropriate subset of methods should be selected and justified for determination of purity.

- **Impurities**

In addition to evaluating the purity of the drug substance and drug product, which may be composed of the desired product and multiple product-related substances, the manufacturer should also assess impurities which may be present. Impurities may be either process- or product-related. They can be of known structure, partially characterized, or unidentified. When adequate quantities of impurities can be generated, these materials should be characterized to the extent possible and, where possible, their biological activities should be evaluated.

Process-related impurities encompass those that are derived from the manufacturing process, i.e., cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (see appendix.B). Product-related impurities (e.g., precursors, certain degradation products) are molecular variants arising during manufacture and/or storage that do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

Further, the acceptance criteria for impurities should be based on data obtained from lots used in preclinical and clinical studies and manufacturing consistency lots.

Individual and/or collective acceptance criteria for impurities (product-related and process-related) should be set, as appropriate. Under certain circumstances, acceptance criteria for selected impurities may not be necessary (section II.C).

Examples of analytical procedures that may be employed to test for the presence of impurities are listed in appendix B. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

For the purpose of lot release (section IV), an appropriate subset of these methods should be selected and justified.

- **Contaminants**

Contaminants in a product include all adventitiously introduced materials not intended to be part of the manufacturing process, such as chemical and biochemical materials (e.g., microbial proteases) and/or microbial species. Contaminants should be strictly avoided and/or suitably controlled with appropriate in-process acceptance criteria or action limits for drug substance or drug product specifications (section II.C). For the special case of adventitious viral or mycoplasma contamination, the concept of action limits is not applicable, and the strategies proposed in ICH guidances *Q5A Quality of Biotechnological/Biological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* and *Q5D Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products* should be considered.

5. *Quantity (2.1.5)*

Quantity, usually measured as protein content, is critical for a biotechnological and biological product and should be determined using an appropriate assay, usually physicochemical in nature. In some cases, it may be demonstrated that the quantity values obtained may be directly related to those found using the biological assay. When this correlation exists, it may be appropriate to use measurement of quantity rather than the measurement of biological activity in manufacturing processes, such as filling.

B. Analytical Considerations (2.2)

1. *Reference Standards and Reference Materials (2.2.1)*

For drug applications for new molecular entities, it is unlikely that an international or national standard will be available. At the time of submission, the manufacturer should have established an appropriately characterized in-house primary reference material, prepared from lot(s) representative of production and clinical materials. In-house working reference material(s) used in the testing of production lots should be calibrated against this primary reference material. Where an international or national standard is available and appropriate, reference materials should be calibrated against it. While it is desirable to use the same reference material for both biological assays and physicochemical testing, in some cases, a separate reference material may be necessary. Also, distinct reference materials for product-related substances, product-related impurities, and process-related impurities may need to be established. When appropriate, a description of the manufacture and/or purification of reference materials should be included in the application. Documentation of the characterization, storage conditions, and formulation supportive of reference material(s) stability should also be provided.

2. *Validation of Analytical Procedures (2.2.2)*

At the time the application is submitted to the regulatory authorities, applicants should have validated the analytical procedures used in the specifications in accordance with the ICH guidances *Q2A Validation of Analytical Procedures: Definitions and Terminology* and *Q2B Validation of Analytical Procedures: Methodology*, except where there are specific issues for unique tests used for analyzing biotechnological and biological products.

C. Process Controls (2.3)

1. *Process-Related Considerations (2.3.1)*

Adequate design of a process and knowledge of its capability are part of the strategy used to develop a manufacturing process that is controlled and reproducible, yielding a drug substance or drug product that meets specifications. In this respect, limits are justified based on critical information gained from the entire process spanning the period from early development through commercial-scale production.

For certain impurities, testing of either the drug substance or the drug product may not be necessary and may not need to be included in the specifications if efficient control or removal to acceptable levels is demonstrated by suitable studies. This testing can include verification at commercial scale in accordance with regional regulations. It is recognized that only limited data may be available at the time of submission of an application. This concept may, therefore, sometimes be implemented after marketing authorization, in accordance with regional regulations.

2. *In-Process Acceptance Criteria and Action Limits (2.3.2)*

In-process tests are performed at critical decision-making steps and at other steps where data serve to confirm consistency of the process during the production of either the drug substance or the drug product. The results of in-process testing may be recorded as action limits or reported as acceptance criteria. Performing such testing may eliminate the need for testing of the drug substance or drug product (section II.C.1). In-process testing for adventitious agents at the end of cell culture is an example of testing for which acceptance criteria should be established.

The use of internal action limits by the manufacturer to assess the consistency of the process at less critical steps is also important. Data obtained during development and validation runs should provide the basis for provisional action limits to be set for the manufacturing process. These limits, which are the responsibility of the manufacturer, may be used to initiate investigation or further

action. They should be further refined as additional manufacturing experience and data are obtained after product approval.

3. Raw Materials and Excipient Specifications (2.3.3)

The quality of the raw materials used in the production of the drug substance (or drug product) should meet standards appropriate for their intended use. Biological raw materials or reagents may require careful evaluation to establish the presence or absence of deleterious endogenous or adventitious agents. Procedures that make use of affinity chromatography (for example, employing monoclonal antibodies) should be accompanied by appropriate measures to ensure that such process-related impurities or potential contaminants arising from their production and use do not compromise the quality and safety of the drug substance or drug product. Appropriate information pertaining to the antibody should be made available.

The quality of the excipients used in the drug product formulation (and in some cases, in the drug substance), as well as the container/closure systems, should meet pharmacopoeial standards, where available and appropriate. Otherwise, suitable acceptance criteria should be established for the nonpharmacopoeial excipients.

D. Pharmacopoeial Specifications (2.4)

Pharmacopoeias contain important requirements pertaining to certain analytical procedures and acceptance criteria which, where relevant, are part of the evaluation of either the drug substance or drug product. Such monographs, applicable to biotechnological and biological products, generally include, but are not limited to, tests for sterility, endotoxins, microbial limits, volume in container, uniformity of dosage units, and particulate matter. With respect to the use of pharmacopoeial methods and acceptance criteria, the value of this guidance is linked to the extent of harmonization of the analytical procedures of the pharmacopoeias. The pharmacopoeias are committed to developing identical or methodologically equivalent test procedures and acceptance criteria.

E. Release Limits Versus Shelf-Life Limits (2.5)

The concept of release limits versus shelf-life limits may be applied where justified. This concept pertains to the establishment of limits which are tighter for the release than for the shelf-life of the drug substance or drug product. Examples where this may be applicable include potency and degradation products. In some regions, the concept of release limits may only be applicable to in-house limits and not to the regulatory shelf-life limits.

F. Statistical Concepts (2.6)

Appropriate statistical analysis should be applied, when necessary, to quantitative data reported. The methods of analysis, including justification and rationale, should be described fully. These descriptions should be sufficiently clear to permit independent calculation of the results presented.

III. JUSTIFICATION OF THE SPECIFICATION (3.0)

The setting of specifications for drug substance and drug product is part of an overall control strategy which includes control of raw materials and excipients, in-process testing, process evaluation or validation, adherence to good manufacturing practices, stability testing, and testing for consistency of lots. When combined in total, these elements provide assurance that the appropriate quality of the product will be maintained. Since specifications are chosen to confirm the quality rather than to characterize the product, the manufacturer should provide the rationale and justification for including and/or excluding testing for specific quality attributes. The following points should be taken into consideration when establishing scientifically justifiable specifications.

- Specifications are linked to a manufacturing process.

Specifications should be based on data obtained from lots used to demonstrate manufacturing consistency. Linking specifications to a manufacturing process is important, especially for product-related substances, product-related impurities, and process-related impurities. Process changes and degradation products produced during storage may result in heterogeneity patterns which differ from those observed in the material used during preclinical and clinical development. The significance of these alterations should be evaluated.

- Specifications should account for the stability of drug substance and drug product.

Degradation of drug substance and drug product, which may occur during storage, should be considered when establishing specifications. Due to the inherent complexity of these products, there is no single stability-indicating assay or parameter that profiles the stability characteristics. Consequently, the manufacturer should propose a stability-indicating profile. The result of this stability-indicating profile will then provide assurance that changes in the quality of the product will be detected. The determination of which tests should be included will be product specific. The manufacturer is referred to the ICH guidance *Q5C Stability Testing of Biotechnological/Biological Products*.

- Specifications are linked to preclinical and clinical studies.

Specifications should be based on data obtained for lots used in preclinical and clinical studies. The quality of the material made at commercial scale should be representative of the lots used in preclinical and clinical studies.

- Specifications are linked to analytical procedures.

Critical quality attributes may include items such as potency, the nature and quantity of product-related substances, product-related impurities, and process-related impurities. Such attributes can be assessed by multiple analytical procedures, each yielding different results. In the course of product development, it is not unusual for the analytical technology to evolve in parallel with the product. Therefore, it is important to confirm that data generated during development correlate with those generated at the time the marketing application is filed.

IV. SPECIFICATIONS (4.0)

Selection of tests to be included in the specifications is product specific. The rationale used to establish the acceptable range of acceptance criteria should be described. Acceptance criteria should be established and justified based on data obtained from lots used in preclinical and/or clinical studies, data from lots used for demonstration of manufacturing consistency, data from stability studies, and relevant development data.

In some cases, testing at production stages rather than testing at the finished drug substance or drug product stages may be appropriate and acceptable. In such circumstances, test results should be considered as in-process acceptance criteria and included in the specification of drug substance or drug product in accordance with the requirements of the regional regulatory authorities.

A. Drug Substance Specification (4.1)

Generally, the following tests and acceptance criteria are considered applicable to all drug substances (for analytical procedures, see section II.B.2). Pharmacopoeial tests (e.g., endotoxin detection) should be performed on the drug substance, where appropriate. Additional drug substance specific acceptance criteria may also be necessary.

1. Appearance and Description (4.1.1)

A qualitative statement describing the physical state (e.g., solid, liquid) and color of a drug substance should be provided.

2. Identity (4.1.2)

The identity test(s) should be highly specific for the drug substance and should be based on unique aspects of its molecular structure and/or other specific properties. More than one test (physicochemical, biological, and/or immunochemical) may be necessary to establish identity. The identity test(s) can be qualitative in nature. Some of the methods typically used for characterization of the product as described in section II.A and in appendix A may be employed and/or modified as appropriate for the purpose of establishing identity.

3. Purity and Impurities (4.1.3)

The absolute purity of biotechnological and biological products is difficult to determine and the results are method dependent (section II.A.4). Consequently, the purity of the drug substance is usually estimated by a combination of methods. The choice and optimization of analytical procedures should focus on the separation of the desired product from product-related substances and from impurities.

The impurities observed in these products are classified as process-related and product-related:

- Process-related impurities (section II.A.4) in the drug substance may include cell culture media, host cell proteins, DNA, monoclonal antibodies or chromatographic media used in purification, solvents, and buffer components. These impurities should be minimized by the use of appropriate, well-controlled manufacturing processes.

- Product-related impurities (section II.A.4) in the drug substance are molecular variants with properties different from those of the desired product formed during manufacture and/or storage.

For the impurities, the choice and optimization of analytical procedures should focus on the separation of the desired product and product-related substances from impurities. Individual and/or collective acceptance criteria for impurities should be set, as appropriate. Under certain circumstances, acceptance criteria for selected impurities may not be necessary (section II.C).

4. Potency (4.1.4)

A relevant, validated potency assay (section II.A.2) should be part of the specifications for a biotechnological or biological drug substance and/or drug product. When an appropriate potency assay is used for the drug product (section IV.B.4), an alternative method (physicochemical and/or biological) may suffice for quantitative assessment at the drug substance stage. In some cases, the measurement of specific activity may provide additional useful information.

5. Quantity (4.1.5)

The quantity of the drug substance, usually based on protein content (mass), should be determined using an appropriate assay. The quantity determination may be independent of a reference standard or material. In cases where product manufacture is based upon potency, there may be no need for an alternate determination of quantity.

B. Drug Product Specification (4.2)

Generally, the following tests and acceptance criteria are considered applicable to all drug products. The sections (IV.B.1 to IV.B.5) are cross-referenced to respective sections (IV.A.1 to IV.A.5) under Drug Substance Specification. Pharmacopoeial requirements apply to the relevant dosage forms. Typical tests found in the pharmacopoeia include, but are not limited to, sterility, endotoxin, microbial limits, volume in container, particulate matter, uniformity of dosage units, and moisture content for lyophilized drug products. If appropriate, testing for uniformity of dosage units may be performed as in-process controls, and corresponding acceptance criteria are set.

1. Appearance and Description (4.2.1)

A qualitative statement describing the physical state (e.g., solid, liquid), color, and clarity of the drug product should be provided.

2. Identity (4.2.2)

The identity test(s) should be highly specific for the drug product and should be based on unique aspects of its molecular structure and other specific properties. The identity test(s) can be qualitative in nature. While it is recognized that in most cases a single test is adequate, more than one test (physicochemical, biological, and/or immunochemical) may be necessary to establish identity for some products. Some of the methods typically used for characterization of the product, as described in section II.A and in appendix A, may be employed and/or modified as appropriate for the purpose of establishing identity.

3. Purity and Impurities (4.2.3)

Impurities may be generated or increased during manufacture and/or storage of the drug product. These may be either the same as those occurring in the drug substance itself, process-related, or degradation products which form specifically in the drug product during formulation or during storage. If impurities are qualitatively and quantitatively (i.e., relative amounts and/or concentrations) the same as in the drug substance, testing is not considered necessary. If impurities are known to be introduced or formed during the production and/or storage of the drug product, the levels of these impurities should be determined and acceptance criteria established.

Acceptance criteria and analytical procedures should be developed and justified, based upon previous experience with the drug product, to measure changes in the drug substance during the manufacture and/or storage of the drug product.

The choice and optimization of analytical procedures should focus on the separation of the desired product and product-related substances from impurities including degradation products, and from excipients.

4. Potency (4.2.4)

A relevant, validated potency assay (section II.A.2) should be part of the specifications for a biotechnological and biological drug substance and/or drug product. When an appropriate potency assay is used for the drug substance, an alternative method (physicochemical and/or biological) may suffice for quantitative assessment of the drug product. However, the rationale for such a choice should be provided.

5. Quantity (4.2.5)

The quantity of the drug substance in the drug product, usually based on protein content (mass), should be determined using an appropriate assay. In cases where product manufacture is based upon potency, there may be no need for an alternate determination of quantity.

6. General Tests (4.2.6)

Physical description and the measurement of other quality attributes are often important for the evaluation of the drug product functions. Examples of such tests include pH and osmolarity.

7. Additional Testing for Unique Dosage Forms (4.2.7)

It should be recognized that certain unique dosage forms may need additional tests other than those mentioned above.

Appendix C

GLOSSARY (5.0)

Acceptance criteria: Numerical limits, ranges, or other suitable measures for acceptance of the results of analytical procedures which the drug substance or drug product or materials at other stages of manufacture should meet.

Action limit: An internal (in-house) value used to assess the consistency of the process at less critical steps.

Biological activity: The specific ability or capacity of the product to achieve a defined biological effect. Potency is the quantitative measure of the biological activity.

Contaminants: Any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the drug substance or drug product.

Degradation products: Molecular variants resulting from changes in the desired product or product-related substances brought about over time and/or by the action of, e.g., light, temperature, pH, water, or by reaction with an excipient and/or the immediate container/closure system. Such changes may occur as a result of manufacture and/or storage (e.g., deamidation, oxidation, aggregation, proteolysis). Degradation products may be either product-related substances or product-related impurities.

Desired product: (1) The protein that has the expected structure, or (2) the protein that is expected from the DNA sequence and anticipated post-translational modification (including glycoforms), and from the intended downstream modification to produce an active biological molecule.

Drug product (Dosage form; Finished product): A pharmaceutical product type that contains a drug substance, generally in association with excipients.

Drug substance (Bulk material): The material that is subsequently formulated with excipients to produce the drug product. It can be composed of the desired product, product-related substances, and product- and process-related impurities. It may also contain excipients including other components, such as buffers.

Excipient: An ingredient added intentionally to the drug substance which should not have pharmacological properties in the quantity used.

Impurity: Any component present in the drug substance or drug product that is not the desired product, a product-related substance, or an excipient including buffer components. It may be either process- or product-related.

In-house primary reference material: An appropriately characterized material prepared by the manufacturer from a representative lot(s) for the purpose of biological assay and

physicochemical testing of subsequent lots, and against which in-house working reference material is calibrated.

In-house working reference material: A material prepared similarly to the primary reference material that is established solely to assess and control subsequent lots for the individual attribute in question. It is always calibrated against the in-house primary reference material.
Potency: The measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties.

Process-related impurities: Impurities that are derived from the manufacturing process. They may be derived from cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (e.g., processing reagents or column leachables).

Product-related impurities: Molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

Product-related substances: Molecular variants of the desired product formed during manufacture and/or storage which are active and have no deleterious effect on the safety and efficacy of the drug product. These variants possess properties comparable to the desired product and are not considered impurities.

Reference standards: International or national standards.

Specification: A list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product, or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. *Conformance to specification* means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.

APPENDICES (6.0)

A. Appendix for Physiochemical Characterization (6.1)

This appendix provides examples of technical approaches that might be considered for structural characterization and confirmation, and evaluation of physicochemical properties of the desired product, drug substance, and/or drug product. The specific technical approach employed will vary from product to product, and alternative approaches, other than those included in this appendix, will be appropriate in many cases. New analytical technology and modifications to existing technology are continuously being developed and should be utilized when appropriate.

1. Structural Characterization and Confirmation (6.1.1)

a. Amino acid sequence

The amino acid sequence of the desired product should be determined to the extent possible using approaches such as those described in items (b) through (e) and then compared with the sequence of the amino acids deduced from the gene sequence of the desired product.

b. Amino acid composition

The overall amino acid composition is determined using various hydrolytic and analytical procedures and compared with the amino acid composition deduced from the gene sequence for the desired product, or the natural counterpart, if considered necessary. In many cases, amino acid composition analysis provides some useful structural information for peptides and small proteins, but such data are generally less definitive for large proteins. Quantitative amino acid analysis data can also be used to determine protein content in many cases.

c. Terminal amino acid sequence

Terminal amino acid analysis is performed to identify the nature and homogeneity of the amino- and carboxy-terminal amino acids. If the desired product is found to be heterogeneous with respect to the terminal amino acids, the relative amounts of the variant forms should be determined using an appropriate analytical procedure. The sequence of these terminal amino acids should be compared with the terminal amino acid sequence deduced from the gene sequence of the desired product.

d. Peptide map

Selective fragmentation of the product into discrete peptides is performed using suitable enzymes or chemicals, and the resulting peptide fragments are analyzed by high pressure liquid chromatography (HPLC) or other appropriate analytical procedures. The peptide fragments should be identified to the extent possible

using techniques such as amino acid compositional analysis, N-terminal sequencing, or mass spectrometry. Peptide mapping of the drug substance or drug product using an appropriately validated procedure is a method that is frequently used to confirm desired product structure for lot release purposes.

e. Sulfhydryl group(s) and disulfide bridges

If, based on the gene sequence for the desired product, cysteine residues are expected, the number and positions of any free sulfhydryl groups and/or disulfide bridges should be determined, to the extent possible. Peptide mapping (under reducing and nonreducing conditions), mass spectrometry, or other appropriate techniques may be useful for this evaluation.

f. Carbohydrate structure

For glycoproteins, the carbohydrate content (neutral sugars, amino sugars, and sialic acids) is determined. In addition, the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile), and the glycosylation site(s) of the polypeptide chain are analyzed, to the extent possible.

2. Physicochemical Properties (6.1.2)

a. Molecular weight or size

Molecular weight (or size) is determined using size exclusion chromatography, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (under reducing and/or nonreducing conditions), mass spectrometry, and other appropriate techniques.

b. Isoform pattern

This is determined by isoelectric focusing or other appropriate techniques.

c. Extinction coefficient (or molar absorptivity)

In many cases, it will be desirable to determine the extinction coefficient (or molar absorptivity) for the desired product at a particular ultraviolet (UV)/visible wavelength (e.g., 280 nanometers). The extinction coefficient is determined using UV/visible spectrophotometry on a solution of the product having a known protein content as determined by techniques such as amino acid compositional analysis or nitrogen determination. If UV absorption is used to measure protein content, the extinction coefficient for the particular product should be used.

d. Electrophoretic patterns

Electrophoretic patterns and data on identity, homogeneity, and purity can be obtained by polyacrylamide gel electrophoresis, isoelectric focusing, SDS-polyacrylamide gel electrophoresis, Western-blot, capillary electrophoresis, or other suitable procedures.

e. Liquid chromatographic patterns

Chromatographic patterns and data on the identity, homogeneity, and purity can be obtained by size exclusion chromatography, reverse-phase liquid chromatography, ion-exchange liquid chromatography, affinity chromatography, or other suitable procedures.

f. Spectroscopic profiles

The UV and visible absorption spectra are determined as appropriate. The higher-order structure of the product is examined using procedures such as circular dichroism, nuclear magnetic resonance (NMR), or other suitable techniques as appropriate.

B. Appendix for Impurities (6.2)

This appendix lists potential impurities, their sources, and examples of relevant analytical approaches for detection. Specific impurities and technical approaches employed, as in the case of physicochemical characterization, will vary from product to product, and alternative approaches other than those listed in this appendix will be appropriate in many cases. New analytical technology and modifications to existing technology are continuously being developed and should be applied when appropriate.

1. Process-Related Impurities and Contaminants (6.2.1)

These are derived from the manufacturing process (section II.A.4) and are classified into three major categories: Cell substrate-derived, cell culture-derived and downstream-derived.

a. Cell substrate-derived impurities include, but are not limited to, proteins derived from the host organism and nucleic acid (host cell genomic, vector, or total DNA). For host cell proteins, a sensitive assay, e.g., immunoassay, capable of detecting a wide range of protein impurities is generally utilized. In the case of an immunoassay, a polyclonal antibody used in the test is generated by immunization with a preparation of a production cell minus the product-coding gene, fusion partners, or other appropriate cell lines. The level of DNA from the host cells can be detected by direct analysis on the product (such as hybridization techniques). Clearance studies, which could include spiking experiments at the laboratory scale, to demonstrate the removal of cell substrate-derived impurities such as nucleic acids and host cell proteins may sometimes be used to eliminate the need for establishing acceptance criteria for these impurities.

b. Cell culture-derived impurities include, but are not limited to, inducers, antibiotics, serum, and other media components.

c. Downstream-derived impurities include, but are not limited to, enzymes, chemical and biochemical processing reagents (e.g., cyanogen bromide, guanidine, oxidizing and reducing agents), inorganic salts (e.g., heavy metals, arsenic, nonmetallic ion), solvents, carriers, ligands (e.g., monoclonal antibodies), and other leachables.

For intentionally introduced, endogenous, and adventitious viruses, the ability of the manufacturing process to remove and/or inactivate viruses should be demonstrated as described in ICH guidance *Q5A Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin.*

2. *Product-Related Impurities Including Degradation Products (6.2.2)*

The following represents the most frequently encountered molecular variants of the desired product and lists relevant technology for their assessment. Such variants may need considerable effort in isolation and characterization in order to identify the type of modification(s). Degradation products arising in significant amounts during manufacture and/or storage should be tested for and monitored against appropriately established acceptance criteria.

a. Truncated forms. Hydrolytic enzymes or chemicals may catalyze the cleavage of peptide bonds. These may be detected by HPLC or SDS-PAGE. Peptide mapping may be useful, depending on the property of the variant.

b. Other modified forms. Deamidated, isomerized, mismatched S-S linked, oxidized, or altered conjugated forms (e.g., glycosylation, phosphorylation) may be detected and characterized by chromatographic, electrophoretic, and/or other relevant analytical methods (e.g., HPLC, capillary electrophoresis, mass spectroscopy, circular dichroism).

c. Aggregates. The category of aggregates includes dimers and higher multiples of the desired product. These are generally resolved from the desired product and product-related substances and quantitated by appropriate analytical procedures (e.g., size exclusion chromatography, capillary electrophoresis).

Appendix D

GUIDANCE FOR INDUSTRY

FOR THE SUBMISSION OF CHEMISTRY, MANUFACTURING, AND CONTROLS INFORMATION FOR A THERAPEUTIC RECOMBINANT DNA-DERIVED PRODUCT OR A MONOCLONAL ANTIBODY PRODUCT FOR IN VIVO USE

Center for Biologics Evaluation and Research (CBER)
Center for Drug Evaluation and Research (CDER)
August 1996

Appendix D

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Appendix D

GUIDANCE FOR INDUSTRY[1]

FOR THE SUBMISSION OF
CHEMISTRY, MANUFACTURING, AND CONTROLS INFORMATION
FOR A THERAPEUTIC RECOMBINANT DNA-DERIVED PRODUCT OR
A MONOCLONAL ANTIBODY PRODUCT FOR IN VIVO USE

I. INTRODUCTION

In the Federal Register of May 14, 1996, the Food and Drug Administration published the final rule ?Elimination of the Establishment License Application for Specified Biotechnology and Specified Synthetic Biological Products". Under this rule manufacturers of therapeutic recombinant DNA-derived products and/or monoclonal antibody products for in vivo use are no longer required to submit an Establishment License Application and may use the interim FDA Form 3439. This document provides guidance on the content and format of the Chemistry, Manufacturing, and Controls (CMC) section of a Biologics License Application for therapeutic recombinant DNA-derived products and monoclonal antibody products for in vivo use.

II. DRUG SUBSTANCE

The drug substance is defined as the unformulated active substance which may be subsequently formulated with excipients to produce the drug product.

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[0] 0 [0]This guidance is an informal communication under 21 CFR 10.90(b)(9) that represents the best judgement of employees of the Center for Biologics Evaluation and Research (CBER) and the Center for Drug Evaluation and Research (CDER), at this time. This statement does not necessarily represent the formal position of CBER or CDER and does not bind or otherwise obligate CBER or CDER to the views expressed. For further information about this guidance, contact Neil Goldman, Ph.D., Associate Director for Research, Center for Biologics Evaluation and Research, 4401 Rockville Pike, Rockville, MD 20852 (Phone: 301-827-0375; Fax: 301-827-0440) or Yuan-Yuan Chiu, Ph.D., Supervisory Chemist, Biotechnology Subcommittee, Center for Drug Evaluation and Research, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-443-3510; Fax: 301-443-9282).

A. Description and Characterization

1. Description

A clear description of the drug substance should be provided. This description may include, but not be limited to, any of the following: chemical structure, primary and subunit structure, molecular weight, molecular formula, established USAN name, antibody class/subclass (if appropriate), etc.

2. Characterization / Proof of Structure

a. Physicochemical Characterization of Reference Standard and Qualifying Lots:

A description and the results of all the analytical testing performed on the manufacturer's reference standard lot and qualifying lots to characterize the drug substance should be included (See references 7, 8, 10). Information from specific tests regarding identity, purity, stability and consistency of manufacture of the drug substance should be provided. Examples of analyses for which information may be submitted include, but are not necessarily limited to the following:

- amino acid analysis
- amino acid sequencing, entire sequence or amino- and carboxy-terminal sequences
- peptide mapping
- determination of disulfide linkage
- Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (reduced and non-reduced)
- isoelectric focusing
- Conventional and High Pressure Liquid Chromatography (HPLC) e.g., reverse-phase, size exclusion, ion-exchange, etc.
- mass spectroscopy

- assays to detect product-related proteins including deamidated, oxidized, cleaved, and aggregated forms and other variants e.g., amino acid substitutions, adducts/derivatives.
- assays to detect residual host proteins, DNA, reagents
- immunochemical analyses
- assays to quantitate bioburden, endotoxin

Additional physicochemical characterization may be required for products undergoing post-translational modifications, for example, glycosylation, sulfation, phosphorylation, or formylation. Additional physicochemical characterization may also be required for products derivatized with other agents, including other proteins, toxins, drugs, radionuclides, or chemicals. The information submitted should include the degree of derivatization or conjugation, the amount of unmodified product, removal of free materials (e.g., toxins, radionuclides, linkers, etc.), and the stability of the modified product.

All test methods should be fully described and the results provided. The application should also include the actual data such as legible copies of chromatograms, photographs of SDS-PAGE or agarose gel, spectra, etc.

b. Biological Activity

A description and results of all relevant <u>in vivo</u> and <u>in vitro</u> biological testing performed on the manufacturer's reference standard lot to show the potency and activity(ies) of the drug substance should be provided. Results of relevant testing performed on lots other than the reference standard lot, that might have been used in establishing the biological activity of the

product, should also be included. (See references 7 - 10, 12) The description and validation of the bioassays should include the methods and standards used, the inter- and intra-assay variability, and the acceptable limits of the assay.

B. Manufacturer(S)

1. Identification

The application should include the name(s), address(es), FDA registration number, and other pertinent organizational information for each manufacturer performing any portion of the manufacture or testing operations for the drug substance. This may include contractors or company subsidiaries serving as contractors, or other locations/sites owned and operated by the applicant. A brief description of the operations performed at each location, the responsibilities conferred upon each party by the applicant and a description of how the applicant will ensure that each party fulfills their responsibilities should be submitted.

2. Floor Diagram(s)

For each manufacturing location, a floor diagram should be included that indicates the general facility(ies) layout. This diagram need not be a detailed engineering schematic or blueprint, but rather a simple drawing that depicts the relationship of the subject manufacturing areas, suites, or rooms to one another, and should indicate other uses made of adjacent areas that are not the subject of the application. This diagram should be sufficiently clear such that the reviewer may visualize the flow of the production of the drug substance and would be able to identify areas or room "proximities" that may be of concern for particular operations, e.g. segregation of pre and post viral inactivation material and operations, segregation of animal facilities, etc. Room numbers or other unique identifiers should be provided, however it is not necessary to include the location of processing equipment within rooms and areas. Reference can be made to manufacturing flow information presented in response to section II. C. 2. of this guidance.

3. Other Products

A comprehensive list of all additional products to be manufactured or manipulated in the areas used for the product should be provided. The applicant should indicate in which rooms the additional products will be introduced and the manufacturing steps that will take place in the room. An explanation should be given as to whether these additional products will be introduced on a campaign basis or concurrently during production of the product which is the subject of the application. Any additional products that may share product contact equipment with the product in question should be indicated (dedicated vs. multi-use equipment should be delineated for each process step, in this section or other appropriate sections of the application). A brief description should be provided as to the type and developmental status of the additional products.

4. Contamination Precautions

For all areas in which operations for the preparation of cell banks and product manufacturing are performed, including areas for the handling of animals used in production, the following information concerning precautions taken to prevent contamination or cross-contamination should be provided:

- air quality classification of room or area in which operation is performed, as validated and measured during operations;
- a brief, narrative description of the procedures and/or facility design features for the control of contamination, cross contamination and containment (air pressure cascades, segregation of operations and product, etc.) - this is of particular importance for multi-use areas or for work with live organisms;
- general equipment design description, eg. does design represent an open or closed system or provide for a sterile or non-sterile operation, and;
- a description of the in-process controls

performed to prevent or to identify contamination or cross contamination.

The manipulation of more than one cell line in a single area, or the use of any piece of equipment for more than one cell line, should be indicated and measures to ensure prevention of cross contamination should be discussed.

C. Method(S) of Manufacture

1. Raw Materials and Reagents

A list of all components used in the manufacture of the drug substance, and their tests and specifications or reference to official compendia should be provided. For purchased raw materials representative certificates of analysis from the supplier(s) and/or manufacturer's acceptance criteria should be included in the submission. Process gases (e.g., air, carbon dioxide) and water are considered raw materials.

A list with tests and specifications of all special reagents and materials used in the manufacture of the drug substance, e.g., culture media, buffers, sera, antibiotics, monoclonal antibodies, preservatives, should be submitted. In cases where an ancillary biological product is used in the manufacture of the drug substance (e.g., a monoclonal antibody used in affinity chromotagraphy), a detailed description of the preparation and characterization of the reagent should be submitted (Reference 7, 10).

A description of the tests and specifications for materials of human or animal source that may potentially be contaminated with adventitious agents, e.g., mycoplasma, Bovine Spongioform Encephalopathy (BSE) agent for bovine derived products, and other adventitious agents of human and animal origin should be submitted. Validation data or certification supporting the freedom of reagents from adventitious agents should be included in the submission.

2. Flow Charts

A complete visual representation of the manufacturing process flow should be provided. This flow chart should indicate the step in production, the equipment and materials used, the room or area where the operation is performed (may reference the simple diagram in II. B. 2.) and a complete list of the in-process controls and tests performed on the product at each step. This diagram should also include information (or be accompanied by a descriptive narrative) on the methods used to transfer the product between steps, i.e. Sterile, steam-in-place (SIP) connection, sanitary connection, open transfers under laminar flow units etc. Such transfers should be described for movement of product between equipment, areas/rooms, buildings and sites. Manufacturing steps which are computer controlled should be identified. References can be made to other sections of the application for more detailed process information.

3. Detailed Description

a. Animal Sources

The information submitted concerning animals used in production, such as mice used for ascites or transgenic animals, should include detailed descriptions of the following:

- source of animals;
- method of creating and the genetic stability of transgenic animals;
- adventitious agent screening and quarantine procedures used to assure animals are appropriate for use in manufacturing;
- animal husbandry procedures, and;
- veterinary oversight.

More detailed guidance in these areas may be obtained from the ?Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" and the ?Points to Consider in the Manufacture and

Testing of Therapeutic Products for Human Use Derived from Transgenic Animals."

b. Cellular Sources

i. Cell Substrate / Host Cell / Expression Vector System

A. Recombinant DNA Products including rDNA-Derived Monoclonal Antibodies.

The submission should include a detailed description of the host cell and expression vector system and their preparation as delineated below: (See References 9, 10, 12)

I. Host Cells

A description of the source, relevant phenotype, and genotype should be provided for the host cell used to construct the biological production system. The results of the characterization of the host cell for phenotypic and genotypic markers, including those that will be monitored for cell stability, purity, and selection should be included.

II. Gene Construct

A detailed description of the gene which was introduced into the host cells, including both the cell type and origin of the source material, should be provided. A description of the method(s) used to prepare the gene construct

and a restriction enzyme digestion map of the construct should be included. The complete nucleotide sequence of the coding region and regulatory elements of the expression construct, with translated amino acid sequence, should be provided, including annotation designating all important sequence features.

III. Vector

Detailed information regarding the vector and genetic elements should be provided, including a description of the source and function of the component parts of the vector, e.g. origins of replication, antibiotic resistance genes, promoters, enhancers. A restriction enzyme digestion map indicating at least those sites used in construction of the vector should be provided. The genetic markers critical for the characterization of the production cells should be indicated.

IV. Final Gene Construct

A detailed description should be provided of the cloning process which resulted in the final recombinant gene construct. The information should include a step-by-step description of the assembly

of the gene fragments and vector or other genetic elements to form the final gene construct. A restriction enzyme digestion map indicating at least those sites used in construction of the final product construct should be provided.

v. **Cloning and Establishment of the Recombinant Cell Lines.**

Depending on the methods to be utilized to transfer a final gene construct or isolated gene fragments into its host, the mechanism of transfer, copy number, and the physical state of the final construct inside the host cell (i.e. integrated or extrachromosomal), should be provided. In addition, the amplification of the gene construct, if applicable, selection of the recombinant cell clone, and establishment of the seed should be completely described.

B. Monoclonal Antibodies.

A detailed description of the development of the monoclonal antibody should be provided including characterization of the parent cells, donor history for human cells, immunogen, immortalization procedures, screening, and cell cloning procedures. (See Reference 7, 9)

ii. Cell Seed Lot System

A. Master Cell Bank (MCB)

A detailed description of the preparation and testing of the MCB, as outlined below and in the ICH guideline ?Analysis of the Expression Construct in Cells used for Production of R-DNA Derived Protein Products", should be submitted.

The MCB should be described in detail, including methods, reagents and media used, date of creation, quantity of the cell bank, in-process controls, and storage conditions. The results of the characterization of the MCB for identity and purity using appropriate phenotypic markers such as morphology, auxotrophy, isoenzyme, etc. should be included. Restriction enzyme analysis and DNA sequencing data supporting the integrity of the introduced genetic sequence and data supporting the stability of both the host cell and final gene construct during storage should also be submitted. For bacterial cells, the results of tests for contamination with both lytic and lysogenic bacteriophages and

non-host microorganism(s) should be included.

The testing of the MCB for endogenous and adventitious agents (e.g. Murine retroviruses, Epstein-Barr virus, mycoplasma, bacteria, fungi, other viruses and/or virus-like particle), as appropriate, as outlined in "Points to Consider in Characterization of Cell Lines used to Produce Biological Products", 1993, and ?Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" should be described. If new Master Cell Banks are to be generated by transfer of final DNA construct to host cells or by expansion of an existing MCB or Working Cell Bank (WCB), then acceptance criteria for both the new cell bank and the drug substance(s) produced by the new bank should be described. In particular, documentation of the fidelity of the introduced nucleotide sequence in the new MCB and restriction mapping analysis should be submitted.

B. **Working Cell Bank**

A detailed description of the preparation and testing of the WCB such as those outlined in the applicable guidance documents (References 7, 9, 10, 12) should be submitted.

The production of the Working Cell Bank should be described in detail, including methods, reagents and media used, date of creation, quantity of the cell

bank, number of cell doublings from the MCB and storage conditions. If there is no MCB, the results of the characterization of the WCB should be provided in the format detailed for the MCB (Section II. C. 3. b. ii.).

c. **End of Production Cells (EPC)**

A detailed description of the characterization of the EPC that demonstrates that the biological production system is consistent during growth should be provided. The results of the analysis of the EPC for phenotypic or genotypic markers to confirm identity and purity should be included. This section should also contain the results of testing supporting the freedom of the EPC from contamination by adventitious agents. The results of restriction enzyme analysis of the gene constructs in the EPC should be submitted. Further guidance can be obtained from the ICH document on ?Analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products" and ?Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use."

iii. **Cell Growth and Harvesting**

A detailed description of the process of inoculation, cell growth and harvesting should be submitted. The composition of the medium, equipment preparation and sterilization, as well as fermentation medium sterilization, should be described. For all stages

of any fermentation process the procedures which prevent contamination with adventitious agents should be described.

The stages of cell growth should be described in detail including the selection of inoculum, scale-up for propagation, and established and proposed (if different) production batch size. All operating conditions and in-process controls should also be described and appropriate ranges for operating and control parameters, such as fermentation time, cell doubling time, cell culture purity, cell viability, pH, CO_2, etc., established. If induction is required for production of protein, detailed information including induction conditions and controls employed should also be described.

The submission should include the process used to inactivate cells utilized in the production of a drug substance prior to their release into the environment. For cell lines meeting the criteria of Good Large Scale Practice (GLSP) organisms (July 18, 1991 FR notice, Vol. 56, no 138, p. 33174), which do not require inactivation prior to release into the environment, the information supporting their qualification as GLSP organisms should be provided. A description of the procedures used, in the event of a contamination, to inactivate a GLSP culture prior to release should be included.

If the culture supernatant or cell pellet is stored prior to processing, data supporting its stability during storage should be provided.

The manipulation of more than one cell line in a single area or the use of

any piece of equipment for more than one cell line should be indicated and measures to ensure prevention of cross contamination should be discussed.

c. **Purification and Downstream Processing**

A detailed description of the purification and downstream processing, including a rationale for the chosen methods, and the precautions taken to assure containment and prevention of contamination or cross contamination should be provided. In-process bioburden and endotoxin limits should be specified where appropriate. Any reprocessing using a validated reprocessing method and the conditions for batch eligibility should be described.

If applicable, indication (or reference to II. B. 2.) should be made as to the multi-use nature of areas and equipment (e.g. campaigning vs. concurrent manufacture; dedicated vs. shared equipment) used for these procedures. A brief description of the controls employed to ensure segregation and prevent cross contamination, or reference to another section containing this information, should be provided.

4. **Batch Records**

A completed (executed) representative batch record of the process of production of the drug substance should be submitted.

D. Process Controls

1. In-Process Controls

A description of the methods used for in-process controls, e.g., those involved in fermentation, harvesting and downstream processing, should be provided.

2. Process Validation

A description and documentation of the validation studies should be provided. If the process was changed or scaled up for commercial production and involved changes in the fermentation steps, the re-validation of cell line stability during growth should be described, as in the previous section, and the data and results provided.

a. Validation Studies for the Cell Growth and Harvesting Process.

A description and documentation of the validation studies which identify critical parameters to be used as in-process controls, to ensure the success of routine production should be submitted. Reference may be made to the flow diagram(s) as appropriate.

b. Validation Studies for the Purification Process.

A description and documentation of the validation of the purification process to demonstrate adequate removal of extraneous substances such as chemicals used for purification, column contaminants, endotoxin, antibiotics, residual host proteins, DNA, and viruses, where appropriate, should be provided. (See references 4, 7 - 10)

c. Microbiology.

A description and documentation of the validation studies for any processes used for media sterilization, inactivating cells prior to their release to the environment, if such inactivation is required, etc., should be provided. If the drug substance is intended to be sterile, information should be submitted as described in the ?Guidance for Industry for the Submission of Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products."

E. Reference Standard(s)

1. Primary Reference Standard

If an international reference standard (WHO, NIBSC) or compendial reference standard (USP)is used, submit the citation for the standard and a certificate of analysis. If no biological potency or chemical reference standard exists, and the applicants establish their own primary reference standard, a description of the characterization, and specifications of the standard should be provided. Submit the results of testing, such as physicochemical and biologic activity determinations, of the standard and provide a certificate of analysis. The Standard Operating Procedures (SOPs) to be used for qualifying a new reference standard should be included. Information should also be provided on the stability of any reference standard.

2. Working Reference Standard (if used).

If an in-house working reference standard is used, a description of the preparation, characterization, specifications, testing and results should be provided. The data from the calibration of the in-house working reference standards against a primary reference standard should also be submitted.

F. Specifications / Analytical Methods

1. Drug Substance Specifications and Tests.

a. Specifications and analytical methods used for release testing, shelf life and distribution should be described.

Specifications and tests for the drug substance sufficient to assure its identity, purity, strength and/or potency, as well as lot-to-lot consistency should be submitted. (See references 3, 4, 7 - 11, 13) Validation of the analytical systems and the data should be provided for non-compendial methods to demonstrate the system suitability.

b. Certificates of Analysis and Analytical Results

Certificates of analysis and analytical results for at least three consecutive qualification lots of the drug substance should be submitted.

2. Impurities Profile.

A discussion of the impurities profiles, with supporting analytical data, should be provided. Profiles of variants of the protein drug substance (e.g., cleaved, aggregated, deamidated, oxidized forms, etc.), as well as non-product related impurities (e.g., process reagents and cell culture components), should be included.

G. Container/Closure System(S)

A description of the container and closure system, and its compatibility with the drug substance should be submitted. The submission should include detailed information concerning the supplier, address, and the results of compatibility, toxicity and biological tests. Alternatively, a Drug Master File (DMF) may be referenced for this information. If the drug substance is intended to be sterile,

evidence of container and closure integrity for the duration of the proposed expiry period should be provided.

H. Drug Substance Stability

A description of the storage conditions, study protocols and results supporting the stability of the drug substance should be submitted in this section. (Refer to ICH document ?Stability Testing of Biotechnological/Biological Products" or other FDA documents such as " Guideline for Submitting Documentation for the Stability of Human Drug and Biologics" for specific information.)

Data from tests to monitor the biological activity and degradation products such as aggregated, deamidated, oxidized, and cleaved forms should be included, as appropriate. Data supporting any proposed storage of intermediate(s) should also be provided.

III. DRUG PRODUCT

A. **Composition**, including components.

A tabulated list of all components with their unit dose and batch quantities for the drug product or diluent in accordance with the ?Guideline for Submitting Documentation for the Manufacture of and Controls for Drug Products" should be submitted. The composition of all ancillary products that might be included in the final product should be included.

B. Specifications & Methods for Drug Product Ingredients

1. Drug Substances Including All Active Ingredients and Ancillary Components.

This section should contain a description of tests and specifications for all active ingredients, if not specified in the Drug Substance section. The specifications for all ancillary products that are included in this product should be provided.

2. Excipients:

Information on all excipients including process gases and water should be included.

a. Compendial Excipient(s).

A list of compendial excipients and the citations for each should be submitted.

b. Non-Compendial Excipient(s).

Tests and specifications should be described. For a novel excipient, the description should include its preparation, characterization, and controls. For inactive ingredients of human or animal origin, certification, results of testing or other procedures, or validation data demonstrating their freedom from adventitious agents should be provided.

C. Manufacturer(s)

The name(s) and address(s) of all manufacturers involved in the manufacture and testing of the drug product including contractors, and a description of the responsibility(ies) of each should be submitted. A list of all other products (research & development, clinical or approved) made in the same rooms should be provided. See II. B. 3. of this document for detailed guidance.

D. Methods of Manufacture and Packaging

A complete description of the manufacturing process flow of the formulated bulk and finished drug product should be provided. This discussion should include a description of sterilization operations, aseptic processing procedures, lyophilization, and packaging procedures. Accompanying this narrative, a flow chart should be provided that indicates the production step, the equipment and materials used, the room or area where the operation is performed (may reference the simple diagram in II. B. 2.) and a listing of the in-process controls and tests performed on the product at each step. This flow

diagram or narrative should also include information on the methods of transfer of the product between steps, i.e. Sterile, SIP connection, sanitary connection, open transfers under laminar flow units, etc. Such transfers should be described for movement of product between equipment, areas/rooms, buildings and sites. References can be made to other sections of the application for more detailed process information.

E. Specifications & Test Methods for Drug Product

1. Sampling Procedures

The sampling procedures for monitoring a batch of finished drug product should be included.

2. Specifications & Methods.

A description of all test methods selected to assure the identity, purity, strength and/or potency, as well as the lot-to-lot consistency of the finished product and the specifications used for the drug product should be submitted. Certificates of analysis and analytical results for at least three consecutive batches should be provided.

The validation data for system stability for all non-compendial tests should be provided. If compendial methods have to be validated to ensure non-interference of special inactive ingredients, the results of those validation studies should be submitted.

F. Container/Closure System(s)

A description of the container and closure system, and its compatibility with the drug product should be submitted. Detailed information concerning the supplier(s), address(es), and the results of compatibility, toxicity and biological tests should be included. Alternatively, a DMF can be referenced for this information. For sterile product, evidence of container and closure integrity should be provided for the duration of the proposed expiry period.

G. Microbiology

Information should be submitted as described in the ?Guidance for Industry for the Submission of Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products."

H. Drug Product Stability

A description of the storage conditions, study protocols and results supporting the stability of the drug product should be provided. This should include information on the stability of intermediate fluids or formulated bulk under specified holding or shipping conditions, as appropriate. For products administered through pumps or other such delivery devices, data on the stability of the drug product in the delivery system should be provided. Stability data supporting the proposed shelf-life of the reconstituted drug product and for all labeled dilutions should be included. The results of all tests used to monitor biological activity and the presence of degradation products such as aggregated, deamidated, oxidized, cleaved, etc. forms of the drug substance should also be included. (See references 3, 7 - 13)

IV. INVESTIGATIONAL PRODUCT/FORMULATION

A discussion of any differences in formulation, manufacturing process, or site between the clinical trials materials and commercial production batches of drug substance and drug product should be submitted. If there are differences, a complete description of these differences should be included. If an investigational drug formulation was different from that of the to-be-marketed finished product, data to support comparability, bioequivalence and/or pharmacokinetic equivalence of the two formulations should be provided, if appropriate. If the manufacturing process and/or site was different, data from appropriate testing to assess the comparability of the investigational and commercial products should be provided (See reference 6).

V. ENVIRONMENTAL ASSESSMENT

An environmental assessment should be prepared as outlined in 21 CFR Part 25. This submission should include a description of the action that is being considered and should address all the components involved in the manufacture and disposal of the product. A statement of exemption under a Categorical Exclusion may be provided if applicable.

VI. METHOD VALIDATION

Provide information as described in the ?Guideline for Submitting Samples and Analytical Data for Methods Validation."

VII. REFERENCES

Guidelines

1 Guidance for Industry for the Submission of Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products

2 Guideline on Sterile Drug Products Produced By Aseptic Processing.

3 Guideline for Submitting Documentation for the stability of Human Drugs and Biologics

4 Guideline for Submitting Documentation for the Manufacture of and Controls for Drug Products

5 Guideline for Submitting Samples and Analytical Data for Methods Validation

6 FDA Guidance Concerning Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-derived Products

Points To Consider

7 Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (1994)

8 Points to Consider in the Manufacture and Testing of

Therapeutic Products for Human Use Derived from Transgenic Animals, 8/22/95

9 Points to Consider - Characterization of Cell Line used to Produce Biological Products, 7/12/93

10 Points to Consider in the Production and Testing of New Drugs and Biologics Produced by Recombinant DNA Technology, 4/10/85

International Conference on Harmonization (ICH) Guidelines

11 Stability Testing of New Drug Substances and Products, 10/27/93

12 Analysis of the Expression Construct in Cells used for Production of R-DNA Derived Protein Products, 11/28/95

13 Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products, 11/30/95

Appendix E

DRAFT
APRIL 10, 1985

Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology

Office of Biologics Research and Review

Center for Drugs and Biologics

Table of Contents

I. Introduction

This document provides suggestions for evaluating safety, purity, and potency of new drugs and biologics produced by recombinant DNA technology. The suggestions expressed herein are expected to change with time as new knowledge is acquired, and should not be regarded as being either definitive or all-inclusive. Accordingly, the points discussed below should be interpreted as those that manufacturers of such products are generally expected to consider during development of new drugs and biologics, in filing notices of claimed investigational exemptions for new drugs (IND), in new drug applications (NDA), and in license applications.

II. General Considerations

General regulations for biologics (e.g. 21 CFR, Chapter I, Subchapter F) and drugs (e.g. 21 CFR, Chapter I, Subchapters C and D) also pertain to products produced by recombinant DNA technology where applicable. Specific concerns relevant to particular products should be discussed with the appropriate Office on a case-by-case basis. New license applications or new drug applications are required before marketing products made with recombinant DNA technology, even if the active ingredient in the product is thought to be identical in molecular structure to a naturally occurring substance or a previously approved product produced in an established manner.

The production of new drugs and biologics by recombinant DNA technology should generally follow the NIH Guidelines for Research Involving Recombinant DNA Molecules. In addition, manufacturers wishing to export their products from the United States should consult the appropriate guidelines published by agencies such as the World Health Organization, and the National Institute for Biological Standards and Control (United Kingdom).

III. Expression Systems

Recombinant DNA technology involves the systematic arrangement and manipulation of specific segments of nucleic acid for construction of composite molecules which, when placed into an appropriate host environment, will yield a desired product. There are three general methods for obtaining a specific coding segment: (a) reverse transcription of mRNA to complementary DNA; (b) isolation of genomic DNA or RNA; or (c) chemical synthesis.

The manufacturer should provide a description of the method used to prepare the segment coding for the desired product, including both the cell type and origin of the source material. A detailed nucleotide sequence analysis, and a restriction enzyme digestion map of the cloned segment should also be included. If a cloned polynucleotide contains more information than coding sequences, i.e. introns or flanking sequences, then these additional sequences should be adequately characterized.

The construction of the vector used for expression of the cloned nucleotide segment into its respective product should also be described. This description should include a detailed explanation of the source and function of the component parts of the vector, e.g. origins of replication, antibiotic resistance genes, promoters, enhancers, whether or not the product is being synthesized as a fusion protein. A restriction enzyme digestion map indicating at least those sites used in construction of the vector should be provided.

The host cell system which will generate the product is coordinated to fit the expression vector. It is, therefore, important that a description of the source, relevant phenotype and genotype of the host be provided, including

literature references. If the host cell is of mammalian origin then it should be thoroughly characterized.[1] Various methods can be utilized to transfer an expression vector into its host, such as transfection, transduction, infection, microinjection, etc. The mechanism of transfer, copy number, and the physical state of the vector inside, the host cell, integrated or extrachromosomal, should be provided.

IV. Master Cell Bank

The Master Cell Bank is a designated seed lot, from which all subsequent seed lots are made. A seed lot consists of aliquots of a single culture, stored in a manner which gives a reasonable assurance of genetic stability. In most cases, a single host cell containing the expression vector should be cloned to give rise to the Master Cell Bank. The cloning history and methodology should be described. If new Master Cell Banks are to be generated periodically by expression vector transfer and clonal selection, acceptance criteria for both the new clones and the product produced by these clones should be described. The stability of both the host cell and expression vector should be investigated. In particular, the fidelity of the nucleotide sequence encoding the expression product in the Master Cell Bank should be verified. Whenever clonal selection is used to construct a new seed lot, DNA sequence analysis of the coding region should be performed.

In cases in which there is multiple integration into the host cell genome of the DNA sequences expressing the protein product, thus making these sequences difficult to characterize, the mRNA encoding the specific product should be cloned and the anticipated coding sequence of the product should be verified, for each Master Cell Bank.

The identity and purity of the cells in each seed lot should be assured by isoenzyme analysis, auxotrophy, antibiotic resistance, and karyology, as appropriate.

Each seed lot should be characterized for adventitious agents including mycoplasma, bacteria, fungi, viruses, and virus-like particles.[1]

V. Production

The cells used in each production run should be characterized by analysis of relevant phenotypic or genotypic markers, and tested for adventitious agents in samples taken just prior to termination of culture. Additionally, a detailed restriction enzyme digestion map of the expression vector and the nucleotide sequence of the insert encoding the expression product should be determined after full scale culture at least once for each Master Cell Bank.

The procedures and materials used for cell growth and induction of product expression should be described in detail.

Data on the consistency of yield of the product from full-scale culture should be maintained, and criteria for the rejection of culture lots should be established.

Penicillin and other beta-lactam containing antibiotics may derivatize proteins and generally should not be used in production runs because of the risk of hypersensitization in product recipients. Similarly, caution should be exercised in the use of such materials as phenylmethylsulphonylfluoride (PMSF, a protease inhibitor), β-propiolactone, formaldehyde and other protein derivatizing chemicals, since multiple exposure to derivatized proteins may lead to undesirable immune responses in recipients of the final product.

VI. Purification

The methodology of harvesting, extraction, and purification should be

described in detail, and the removal of any undesirable chemicals introduced by these procedures should be demonstrated.

The extent of purification of recombinant DNA products should be consistent with the intended use of the product. Drugs and biologics which are to be administered repeatedly or at high concentrations should be adequately pure to prevent the development of undesired immune or toxic reactions to contaminants. Although recombinant DNA products may be demonstrated to be 99% pure by physicochemical characterization, special attention should be directed toward the removal of certain contaminants which may be present in small amounts. The purification process should be designed to specifically eliminate detectable viruses, microbial and nucleic acid contamination and undesirable antigenic materials.

The use of antibodies for affinity purification of recombinant DNA products deserves special comment. The antibodies should be shown to be free from unwanted biologically active substances such as DNA and viruses as described in Section VII, C.[2] Methods used for the coupling of the antibody to the column matrix and the removal of contaminants from the affinity column should be described. Several production lots of the final product should be examined for the absence of detectable immunoglobulin protein.

VII. Characterization of the Product

Evidence for identity, purity, and stability of the product in comparison with reference preparations may be derived from the results of a wide variety of tests. The specific tests that will adequately characterize any particular product on a lot to lot basis will depend on the nature of the product. Some examples of tests which may be useful during product development or lot to lot testing are described below.

A. Physicochemical Characterization of Proteins

1. Amino Acid Composition Analysis

The complete amino acid composition of the peptide or protein should include accurate values for methionine 1/2-cystine and tryptophan, which may require sample preparation procedures other than hydrolysis in 6N HCl or chemical modification of proteins and analysis of derivitized amino acids. The amino acid composition presented should be the average of at least three (3) separate hydrolysates of each lot number.

For small proteins or peptides with molecular weight less than 10,000, the demonstration of nearly integral ratios of amino acids would support arguments of peptide purity.

For proteins with molecular weight in excess of 10,000 the amino acid composition analysis may not provide as useful information in support of the purity of the product as for the small proteins or peptides. However, integral values for those amino acid residues generally found in low quantities» such as tryptophan and/or methionine, could be obtained and used to support arguments of purity.

2. Partial Sequence Analysis

Where possible, partial amino terminal (15 residues) and carboxy terminal sequence analyses can serve as important criteria for the identity of recombinant DNA produced proteins or peptides. The sequence data presented in tabular form should include the total yield for every amino acid at each cycle, as well as the repetitive yield for the major sequence(s). In several cases. unexpected heterogeneity in the amino termini and carboxy termini of proteins produced by recombinant DNA technology has been observed by using protein sequence analysis.

3. Peptide Mapping

Peptide mapping can provide a very discriminating comparison between a recombinant DNA product and an authentic sample of the natural product or a reference preparation. In conjunction with amino acid composition and sequence analysis of each peptide, peptide mapping can provide precise evidence for the identity of a protein. For proteins containing disulfide bonds, peptide mapping often can be used to verify the correct arrangement of disulfide bonds in the final product.

4. Polyacrylamide Gel Electrophoresis (PAGE) and Isoelectric Focusing

PAGE and isoelectric focusing are valuable techniques for verifying identity, purity and apparent molecular weight of proteins and peptides. The PAGE analysis should include the use of denaturing conditions with and without exposure to reducing agents, and with appropriate molecular weight standards or reference preparations.

It is preferable to analyze samples on slab gels stained by an appropriately sensitive method: for example, silver stain is generally more sensitive than Coomassie blue for the detection of very small quantities of proteins and is useful in identifying nonprotein materials such as nucleic acid, carbohydrate and lipid which may be present.

For peptides of molecular weight less than ca. 8,000, most PAGE methods may not be sufficiently accurate for molecular weight estimates.

5. High Performance Liquid Chromatography (HPLC)

HPLC is a useful method to determine the purity of a protein or peptide, to evaluate its molecular configuration and, under some circumstances, to confirm its identity. HPLC may be especially useful in

characterizing and quantitating specific impurities in the final product, and in peptide mapping.

6. Circular Dichroism and Optical Rotatory Dispersion (CD and ORD)

A comparison of the CD or the ORD spectrum of the material prepared by recombinant DNA technology with the corresponding spectrum of the native material or a reference preparation may support conformational similarity.

7. Other Characterization

Additional physicochemical characterizations may be appropriate for recombinant DNA products containing carbohydrates, DNA, lipids, and other nonprotein components.

B. Biological Tests for Identity and Potency

A comparison of the recombinant DNA product to the natural product or reference preparation in a suitable bioassay will provide additional evidence relating to the identity and potency of the recombinant DNA product. Various types of bioassay may be used. *In vitro* assays are usually faster, less expensive, and more precise than animal studies, yet adequate testing of a biological product may involve animal studies.

Most vaccines produced by recombinant DNA techniques should be compared to the natural substance or reference preparations with respect to their ability to promote immune responses in animals. Non-vaccine recombinant DNA products may be compared with the natural product in pharmacokinetic studies including tissue distribution and clearance mechanism. The extent, frequency and methods of animal testing should be determined on a case-by-case basis.

C. Tests for Contaminants

Reliable and sensitive tests will be needed to assay for trace contamination and product related impurities in the final product on a lot to lot basis. Although physiochemical characterization can ensure a high degree of purity of a recombinant product, tests for trace contaminants will rely heavily on biological indicator systems.

1. Pyrogen Contamination

Pyrogenicity testing should be conducted by injection of rabbits with the final product or by the limulus amebocyte lysate (LAL) assay.[3] Criteria comparable to those adopted for acceptance of the natural product should be used for the recombinant DNA product.

Certain biological pharmaceuticals are pyrogenic in humans despite having passed the LAL test and the rabbit pyrogen test. This phenomenon may be due to materials which appear to be pyrogenic only in humans. To attempt to predict whether human subjects will experience a pyrogenic response, human blood mononuclear cells can be cultured in vitro with the final product and the cell culture fluid injected into rabbits. A fever in the rabbits indicates that the product contains substances which may be pyrogenic in humans.[4]

2. Viral Contamination

Tests for viral contamination should be appropriate to the cell substrate and culture conditions employed.[1] Absence of detectable adventitious viruses contaminating the final product should be demonstrated.

3. Nucleic Acid Contamination

Removal of nucleic acid at each step in the purification process may be demonstrated in pilot experiments by examining the extent of

elimination of added host cell DNA. Such an analysis would provide the theoretical extent of the removal of nucleic acid during purification.

Direct analyses of nucleic acid in several production lots of the final product should be performed by hybridization analysis of immobilized contaminating nucleic acid utilizing appropriate probes, such as both nick-translated host cell and vector DNA. This method ought to provide sensitivity on the order of 10 picograms per dose. Theoretical concerns regarding transforming DNA derived from the cell substrate will be minimized by the general reduction of contaminating nucleic acid.

4. Antigen Contamination

Products which are administered repeatedly or in large doses should be assayed for trace antigenic constituents and product related impurities (e.g. aggregates or degradation products) likely to contaminate the final product. Tests such as Western blots, radioimmunoassays and enzyme-linked immunosorbant assays using high affinity antibodies raised against the product, host cell lysates, appropriate subcellular fractions, and culture medium constituents, should be used to detect contaminating antigens. Because the detection of antigens will be limited by the specificity and sensitivity of the antisera used, these immunoassays will complement but not replace silver stain analysis of SDS-PAGE gels. Patients given large or repeated doses of a product should be monitored for the production of antibodies to contaminating antigens.

5. Microbial Contamination

Appropriate tests should be conducted for microbial contamination that demonstrate the absence of detectable bacteria (aerobes and anaerobes), fungi, yeast and mycoplasma in the final product.

D. Preclinical Toxicity Evaluation

The specific preclinical testing needs are best addressed on a case-by-case basis with the appropriate Office. Appropriate animal tests. which might include those for carcinogenicity, teratogenicity and effects on fertility may be necessary for a product in which the active ingredient is radically altered from the natural substance.

VIII. Modified Protein Products

Using recombinant DNA procedures it may be possible to modify the structure of proteins to enhance their desired biological properties and/or diminish undesirable ones. Any substance that is not a natural constituent of the human body may be antigenic and also may cause unknown and possibly adverse biological effects. The use of such a product in humans depends on a careful assessment of its new benefits compared to the risks identifiable during its preclinical and clinical evaluation.

IX. Clinical Trials

Clinical trials will be necessary for products derived from recombinant DNA technology to evaluate their safety and efficacy.

[1]See "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals." Office of Biologics Research and Review, Center for Drugs and Biologics. FDA. (Federal Register, Vol. 49, NO. 110. June 6, 1984).

[2]See "Points to Consider in the Manufacture of Injectable Monoclonal Antibody Products Intended for Human Use In Vivo," Office of Biologics Research and Review, Center for Drug and Biologics, FDA. (Federal Register, Vol. 49, p. 1138, January 9, 1984).

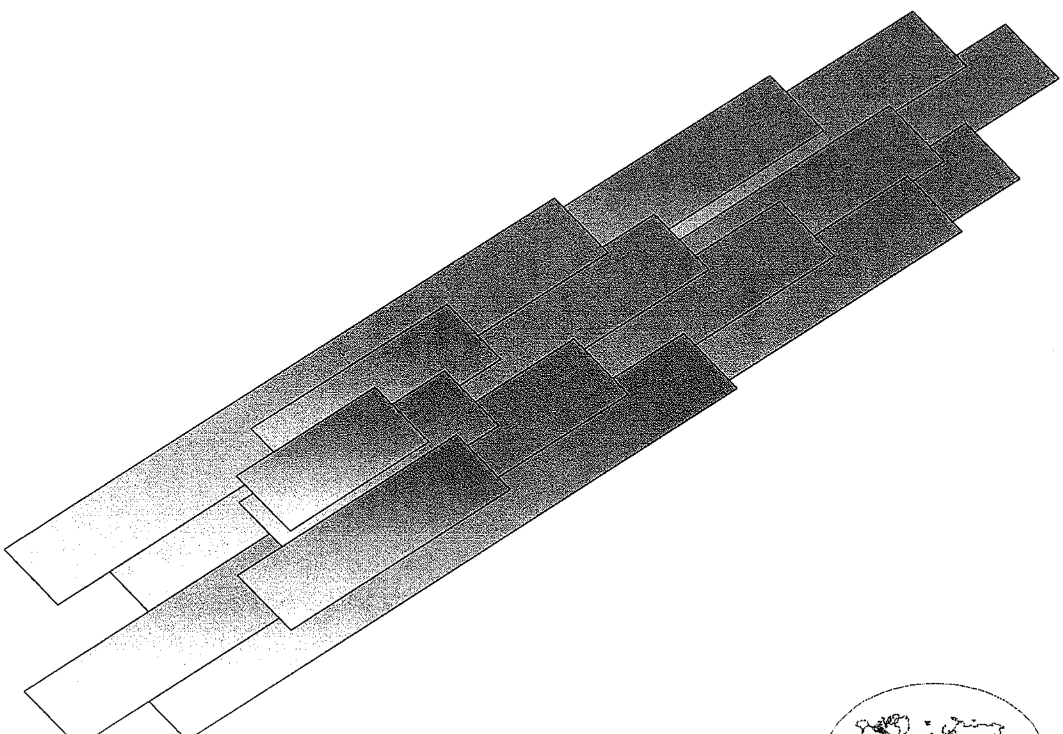
[3]Hochstein. H.D., Elin, R.J., Cooper, J.F., Seligmann, Jr., E.R., and Wolff, S.M. (1973). Bull. Parenteral Drug Assoc., 27, 139-148.

[4]Dinarello. C.A., (1974) "Endogenous pyrogen" in Methods for Studying Mononuclear Phagocytes, Adams. D., Edelsan, P., and Koren, H., Eds., pp. 629-639, Academic Press.

Appendix F

Guideline for Industry

Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products



July 1996

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GUIDELINE FOR INDUSTRY [1]

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

I. INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the

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[1] This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 20, 1995. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the *Federal Register* on July 10, 1996 (61 FR 36466) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on stability testing of biotechnological/biological products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at http://www.fda.gov/cder and go to the "Regulatory Guidance" section).

product's potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

II. SCOPE OF THE ANNEX (2)

The guidance stated in this annex to "Stability Testing of New Drug Substances and Products" applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

III. TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the "Glossary" in "Stability Testing of New Drug Substances and Products." However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

IV. SELECTION OF BATCHES (4)

A. Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification

may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

B. Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

C. Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those

long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

D. Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

V. STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

A. Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

B. Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable in vivo or in vitro quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, in vitro tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

C. Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely

difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS09Page, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

D. Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

VI. STORAGE CONDITIONS (6)

A. Temperature (6.1)

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

B. Humidity (6.2)

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

C. Accelerated and Stress Conditions (6.3)

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on

stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

D. Light (6.4)

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

E. Container/Closure (6.5)

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

F. Stability after Reconstitution of Freeze-Dried Product (6.6)

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

VII. TESTING FREQUENCY (7)

The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

VIII. SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

IX. LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

X. GLOSSARY (10)

- Conjugated Product

  A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

- Degradation Product

A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products, some degradation products may be active.

- Impurity

Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

- Intermediate

For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.

- Manufacturing Scale Production

Manufacture at the scale typically encountered in a facility intended for product production for marketing.

- Pilot-Plant Scale

The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/ BIOLOGICAL PRODUCTS *)

Guideline Title	Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products *)
Legislative basis	Directive 75/318/EEC as amended
Date of first adoption	December 1995
Date of entry into force	July 1996
Status	Last revised December 1995
Previous titles/other references	ICH Q5C, CPMP/ICH/138/95
Additional Notes	This note for guidance concerns the application of Part 2, sections C and F of the Annex to Directive 75/318/EEC as amended, with a view to the granting of a marketing authorisation for a new medicinal product of biological or biotechnological origin. It is an annex to the *Stability Testing of New Active Substances and Medicinal Products* guideline which should be consulted for basic principles.

CONTENTS

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/ BIOLOGICAL PRODUCTS *)

1. PREAMBLE

The guidance stated in the ICH Harmonised Tripartite Guideline *Stability Testing of New Drug Substances and Products*, hereafter called Tripartite Guideline on Stability, (published in this volume under the title *Stability Testing of New Active Substances and Medicinal Products*) applies in general to biotechnological/biological products. However, biotechnological/biological products do have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products, in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. In order to ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physico-chemical, biochemical and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With the above concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions which can affect the product's potency, purity and quality. Primary data to support a requested storage period for either an active substance or medicinal product should always be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

2. SCOPE OF THE ANNEX

The guidance stated in this annex applies to well-characterised proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumour necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth

factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterised proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins or whole blood.

3. TERMINOLOGY

For the basic terms used in this annex the reader is referred to the "Glossary" in the Tripartite Guideline on Stability. However, since manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

4. SELECTION OF BATCHES

4.1 Active substance (Bulk Material)

Where bulk material is to be stored after manufacture but prior to formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of six months stability data at the time of submission should be submitted in cases where storage periods greater than six months are requested. For active substances with storage periods of less than six months, the minimum amount of stability data in the initial submission should be determined on a case by case basis. Data from pilot-plant-scale batches of active substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of active substance placed into the stability program should be representative of the quality of the material used in pre-clinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the active substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The active substance entered into the stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for active substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

4.2 Intermediates

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. While the use of pilot-

plant-scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing-scale process.

4.3 Medicinal product (Final Container Product)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of six months data at the time of submission should be submitted in cases where storage periods greater than six months are requested. For medicinal products with storage periods of less than six months, the minimum amount of stability data in the initial submission should be determined on a case by case basis. Product expiration dating will be based upon the actual data submitted in support of the application. Since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of medicinal product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in pre-clinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

4.4 Sample selection criteria

Where one product is distributed in batches differing in fill volume (e.g. 1 millilitre (ml), 2 ml, or 10 ml), unitage (e.g. 10 units, 20 units, or 50 units), or mass (e.g. 1 milligram (mg), 2 mg, or 5 mg) samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e. the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same medicinal product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure and possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

5. STABILITY-INDICATING PROFILE

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasised in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to adequately demonstrate product stability.

5.1 Protocol

The dossier accompanying the application for marketing authorisation should include a detailed protocol for the assessment of the stability of both active substance and medicinal product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information which demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the Tripartite Guideline on Stability.

5.2 Potency

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognised standard. Where no national or international standards exists, the assay results may be reported in in-house derived units using a characterised reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active substance(s) to a second moiety or binding to an adjuvant. Dissociation of the active substance(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult since, in some cases, in vitro tests for biological activity and physico-chemical characterisation are impractical or provide inaccurate results. Appropriate strategies (e.g. testing the product prior to conjugation/binding, assessing the release of the active compound from the second moiety, in vivo assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of in vitro testing.

5.3 Purity and Molecular Characterisation

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Due to the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the active substance and medicinal product used in the pre-clinical and clinical studies.

The use of relevant physico-chemical, biochemical and immunochemical analytical methodologies should permit a comprehensive characterisation of the active substance and/or medicinal product (e.g. molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulphoxidation, aggregation or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g. reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated and/or stress stability studies, consideration should be given to potential hazards and to the need for characterisation and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in pre-clinical and clinical studies.

For substances that can not be properly characterised or products for which an exact analysis of the purity cannot be meaningfully determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

5.4 Other Product Characteristics

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the medicinal product in its final container:

- Visual appearance of the product (colour and opacity for solutions/suspensions; colour, texture and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilised cakes, pH, and moisture level of powders and lyophilised products.
- Sterility testing or alternatives (e.g. container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.
- Additives (e.g. stabilisers, preservatives) or excipients may degrade during the dating period of the medicinal product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of

the medicinal product, these items may need to be monitored during the stability program.

- The container/closure has the potential to adversely affect the product and should be carefully evaluated (see below).

6. STORAGE CONDITIONS

6.1 Temperature

Since most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

6.2 Humidity

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

6.3 Accelerated and stress conditions

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the active substance and medicinal product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information for future product development (e.g. preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information which may help elucidate the degradation profile of the active substance or medicinal product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g. during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the active substance or medicinal product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. While the Tripartite Guideline on Stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case by case basis.

6.4 Light

Applicants should consult the appropriate regulatory authorities on a case by case basis to determine guidance for testing.

6.5 Container/Closure

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampoules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

6.6 Stability after Reconstitution of Freeze-Dried Product

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

7. TESTING FREQUENCY

The shelf-lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf-lives for existing products and potential future products will be within the range of 0.5 to five years. Therefore, the guidance is based upon expected shelf-lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf-lives of one year or less are proposed, the real-time stability studies should be conducted monthly for the first three months and at three-month intervals thereafter.

For products with proposed shelf-lives of greater than one year, the studies should be conducted every three months during the first year of storage, every six months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the pre-approval or pre-license stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol which supports elimination of specific test intervals (e.g. nine-month testing) for post-approval/post-licensure, long-term studies.

8. SPECIFICATIONS

Although biotechnological/biological products may be subject to significant losses of activity, physico-chemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physico-chemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case by case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that clinical performance is not affected as discussed in the Tripartite Guideline on Stability.

9. LABELLING

For most biotechnological/biological substances and products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for active substances and medicinal products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national regional requirements.

GLOSSARY

Conjugated Product

A conjugated product is made up of an active substance (for example, peptide, carbohydrate) bound covalently or noncovalently to a carrier (for example, protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product

A molecule resulting from a change in the active substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g. by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products some degradation products may be active.

Impurity

Any component of the active substance (bulk material) or medicinal product (final container product) which is not the chemical entity defined as the active substance, an excipient, or other additives to the medicinal product.

Intermediate

For biotechnological/biological products, a material produced during a manufacturing process which is not the active substance or the medicinal product but whose manufacture is critical to the successful production of the active substance or the medicinal product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step prior to continuation of the manufacturing process. This includes material which may undergo further molecular modification or be held for an extended period of time prior to further processing.

Manufacturing-Scale Production

Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale

The production of the active substance or medicinal product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

PRODUCTION AND QUALITY CONTROL OF MEDICINAL PRODUCTS DERIVED BY RECOMBINANT DNA TECHNOLOGY

Guideline Title	Production and Quality Control of Medicinal Products derived by recombinant DNA Technology
Legislative basis	Directive 75/318/EEC as amended
Date of first adoption	First adopted June 1987 This version adopted December 1994
Date of entry into force	July 1995
Status	Last revised December 1994
Previous titles/other references	III/3477/92
Additional Notes	This note for guidance is intended to facilitate the collection and submission of data to support applications for marketing authorisation within the EEC for polypeptide based products derived by rDNA technology and intended for medicinal use in man. It concerns the application of Part 2, sections A-E of the Annex to Directive 75/318/EEC as amended, with a view to the granting of a marketing authorisation for a new medicinal product derived by rDNA technology.

CONTENTS

PRODUCTION AND QUALITY CONTROL OF MEDICINAL PRODUCTS DERIVED BY RECOMBINANT DNA TECHNOLOGY

1. INTRODUCTION

Developments in molecular genetics and nucleic acid chemistry enable the genes coding for natural, biologically active proteins to be identified, analysed in fine detail, transferred between organisms, and expressed under controlled conditions so as to obtain synthesis of the polypeptide for which they code.

Sufficient quantities of medicinal products which were previously difficult to prepare from natural sources can now be produced using such recombinant DNA (rDNA) technology. In addition, the ability to synthesise and manipulate nucleic acids allows the construction of genes coding for modified products possessing different properties from their natural counterpart, or even entirely novel products.

A common strategy in the development of rDNA derived products is the insertion of naturally occurring or intentionally modified natural sequences or novel nucleotide sequences into a vector which is introduced into a suitable host organism so as to ensure the efficient expression of the desired gene product. Both prokaryotic and eukaryotic vector/host cell expression systems have been developed and are in use for production. The factors affecting the expression of foreign genes introduced into a new host using a suitable vector are complex and the efficient, controlled expression of stable, cloned DNA sequences is an important aspect of product development.

A flexible approach to the control of these products should be adopted so that recommendations can be modified in the light of experience of production and use, and with the further development of new technologies. Implementation of these recommendations for an individual product should reflect its intended clinical use.

This note for guidance is intended to facilitate the collection and submission of data to support applications for marketing authorisation within the European Union for polypeptide based products derived by rDNA technology and intended for medicinal use in man. It should be read in conjunction with the European Directives and other specialised guidelines where appropriate.

2. POINTS TO CONSIDER IN PRODUCTION

Requirements relating to establishments in which biological products are produced (e.g. GMP Directive 91/356/EEC and Directive 90/219/EEC on the contained use of genetically modified micro-organisms) will apply to the production of products derived by rDNA methodology as will several of the general recommendations for the quality control of biological products.

Thus, appropriate attention needs to be given to the quality of all reagents used in production, including components of fermentation media; specifications for these are to be included in documentation and they must comply with any relevant European recommendations (e.g.

note for guidance on *Minimising the Risk of Transmitting Agents causing Spongiform Encephalopathy via Medicinal Products*).

Tests for potency, abnormal toxicity, pyrogenicity and sterility etc., which apply to products made by conventional methods, will also apply to products made by rDNA technology. It is undesirable to use in production agents which are known to provoke sensitivity in certain individuals, such as, for example, penicillin or other ß-lactam antibiotics.

Although comprehensive characterisation of the final product is essential, considerable emphasis must also be placed on "in-process" control, a concept which has been highly effective in the quality control of bacterial and viral vaccines prepared by conventional methods.

Certain factors may compromise the consistency, safety and efficacy of rDNA-derived products; these should be given special attention and are outlined below:

a) All biological systems are inherently subject to genetic alteration through mutation and selection and foreign genes inserted into new host cells may exhibit increased genetic instability. The purpose of molecular genetic studies is to establish that the correct sequence has been made and incorporated in the host cell and that both the structure and the number of copies of the inserted sequence are maintained within the cell during culture to the end of production. Such studies can provide valuable information which should be considered in conjunction with tests performed at the protein level for assuring the quality and consistency of the product.

b) Products expressed in foreign hosts may deviate structurally, biologically or immunologically from their natural counterparts. Such alterations can arise at post-translational level or during production or purification and may lead to undesirable clinical effects. Therefore, their presence must be justified and shown to be consistently controlled.

c) The choice of manufacturing procedure will influence the nature, range and amount of potential impurities in the final product and which the purification processes must be shown to be capable of removing. Examples of these are endotoxins in products expressed in bacterial cells, and adventitious agents and DNA in products expressed in mammalian cells.

d) Unintended variability in the culture during production may lead to changes which favour the expression of other genes in the host/vector system or which cause alteration in the product. Such variation might result in differing yield, in change to the product itself (e.g. in the nature and degree of glycosylation) and/or in quantitative and qualitative differences in the impurities present. Consequently, procedures to ensure consistency of production conditions as well as the final product are imperative.

e) Extensive "scale-up" at the level of fermentation and/or purification occurs as laboratory developments progress to full scale commercial production, and this may have considerable consequences for the quality of the product including effects on its conformational structure, yield and/or in quantitative and qualitative differences in impurities. Therefore, sufficient in-process controls and quality control tests during each production run to show equivalency are required.

Whilst the recommendations set out below should be considered to be generally applicable, individual products may present particular quality control issues. Thus, the production and control of each product must be given careful individual consideration taking fully into account any special features.

3. DEVELOPMENT GENETICS

3.1 Gene of interest, Vector and Host Cell

A detailed description of the cloned gene should be given. This should include details of its origin, identification and isolation, as well as the details of the origin and structure of the expression vector. A description of the host strain or cell line should be provided including the history of the strain or cell line, its identification characteristics and potential viral contaminants. Special attention should be given to the possibility of cross-contamination with other cells or viruses.

3.2 Expression construct

Full details of the nucleotide sequence of the gene of interest and of the flanking control regions of the expression vector should be provided to confirm that the construction is identical to that desired. The steps in the assembly of the expression construct should be described in detail. A detailed map and a complete annotated sequence of functionally relevant regions of the vector should be given, indicating the regions which have been sequenced during the construction and those deduced from the literature. All the junctions created by ligation during construction directly impinging on the expression of the inserted gene should be confirmed by sequencing. All known expressed sequences should be clearly identified.

3.3 Status of the rDNA within the host cell

The method by which the vector is introduced into the host cell and the status of the rDNA within the host (integrated or extrachromosomal, copy number, etc.) should be described. For extrachromosomal expression systems, the percent of host cells retaining the expression construct should be determined. The coding sequence for the recombinant product of the expression construct should be verified at the cell bank stage. In systems where multiple integrated copies of the gene exist, which may or may not be the result of amplification, a detailed study using various restriction enzymes and Southern blot analysis should be used, in addition to sequence analysis of mRNA or cDNA molecules in order to provide convincing data on the integrity of the expressed gene(s).

3.4 Expression

The strategy by which the expression of the relevant gene is promoted and controlled during production should be described in detail.

3.5 Stability of the expression system

The stability of host/vector genetic and phenotypic characteristics should be investigated up to and beyond the population doubling level or generation number used for routine

production (End of Production Cells). The expression construct should be analysed in the End of Production Cells, as described above, at least once for each MCB.

Stability studies should also provide detailed information on:

i) gene copy number in relation to productivity of the culture,

ii) deletions and/or insertions affecting any part of the expression vector

iii) the protein produced.

For this purpose, analysis should be performed in such a way that the results can confirm that the number of variants is below an acceptable limit to be established on a case by case basis depending on the nature and proposed use of the product. Analysis at the protein and/or at the DNA level can be envisaged. Whichever method is used, it should be validated and the detection limit given.

4. CONTROL OF CELL BANKS

It is essential that production is based on a well defined master and working cell bank system. During the establishment of the banks no other cell lines should be handled simultaneously in the same laboratory suite or by the same persons. The origin, form, storage, use, and expected duration at the anticipated rate of use must be described in full for all cell banks. New working cell banks should be fully characterised.

A critical part of quality control will involve the full characterisation of cells. Where eukaryotic cells are used for production, distinguishing genetic, phenotypic and immunological markers of the cell will be useful in establishing the identity of the cells. Likewise, where microbial cultures are used, specific phenotypic features which form a basis for identification should be described.

The cell banks should be examined for adventitious agents (viral, bacterial, fungal and mycoplasmal). Special attention should be given to viruses which can commonly contaminate the animal species from which the cell line has been derived. Certain cell lines contain endogenous viruses, e.g. retroviruses, which may not readily be eliminated. The possibility of mutations of endogenous viruses during prolonged culture should be considered. Furthermore, the purification process should be shown to be capable of removing and/or inactivating any such virus which may inevitably be present in the cells as an endogenous agent.

Cell banks should be periodically tested for cell viability, genetic and phenotypic stability and any other relevant parameters.

5. FERMENTATION OR CELL CULTURE

A clear definition of a "batch" of product for further processing should be provided.

Whatever the production process, details of the fermentation or culture with the in-process controls should be provided. Criteria for rejection of harvests and premature termination of the culture should be defined.

The presence, extent and nature of any microbial contamination in the culture vessels must be thoroughly examined at a suitable stage at the end of each production run. Detailed

information to confirm the adequate sensitivity of the methods used to detect contamination should be provided and acceptable limits of contamination set.

Ideally not more than one cell line should be cultivated simultaneously in the same production area. If other cell lines are cultivated in parallel, records must be kept of the cell lines handled and validation data presented for the absence of cross-contamination between them.

5.1 Single harvest production

The maximum permitted generation number or population doubling level for production should be defined and should be based on information concerning the stability of the host cell/vector system up to and beyond the level of production. Data on consistency of growth of the culture and on the maintenance of yield within specified limits should be presented. Appropriate monitoring of host cell/vector characteristics at the end of the production cycles should also be undertaken. Evidence should be provided that the yield does not vary beyond defined limits and that the nature and quality of the product does not change with respect to specific parameters.

5.2 Multiple harvest production

The period of continuous cultivation should be specified and this should be based on information concerning the stability of the system and consistency of the product up to and beyond this limit. Monitoring of the production system is necessary throughout the duration of the culture. The required frequency and type of monitoring will depend upon several factors including the nature of the expression system and product, as well as the total length of the period of continuous cultivation undertaken. The acceptance of harvests for further processing should be clearly linked to the schedule of monitoring applied. Evidence should be provided that the yield does not vary beyond defined limits and that the nature and quality of the product does not change with respect to specific parameters.

6. PURIFICATION OF THE PRODUCT

6.1 Methods

Methods used to purify the product and their in-process controls including their specification limits should be described in detail, justified and validated. Procedures which make use of affinity chromatography, for example employing monoclonal antibodies, should be accompanied by appropriate measures to ensure that these substances, or any additional potential contaminants arising from their use, do not compromise the quality and safety of the final product. Attention is drawn to the notes for guidance "Production and Quality Control of Monoclonal Antibodies" and "Virus validation studies: The design, contribution and interpretation of studies validating the inactivation and removal of viruses".

The criteria for reprocessing of any intermediate or final bulk should be carefully defined, validated and justified.

6.2 Validation of the purification procedure

The capacity of the purification procedure to remove unwanted host cell derived proteins, nucleic acids, carbohydrates, viruses and other impurities including product-related proteins should be investigated thoroughly.

Studies using a carefully selected group of viruses which exhibit a range of physico-chemical features relevant to their behaviour on purification (see note for guidance *Virus Validation Studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses*) intentionally mixed with the crude preparation (spiking) should be undertaken. The ability of the purification process to remove other specific contaminants such as host-cell proteins, other potential impurities derived from the production process and DNA should also be demonstrated using, where necessary, concentrations of those contaminants in excess of that expected during normal production (spiking). A reduction factor for such contaminants at each stage of purification, and overall, should be established.

Validation of the purification process should also include justification of the working conditions such as column loading capacity , column regeneration and sanitisation and length of use of the columns. Columns should also be validated regarding leaching of ligands (e.g. dye, affinity ligand, etc.) and/or chromatographic material, throughout the expected life span of the column.

7. ACTIVE SUBSTANCE

7.1 Characterisation of the active substance

7.1.1 Physico-chemical characterisation, relative molecular mass, pI value

Rigorous characterisation of the active substance by chemical and biological methods will be essential. Particular attention should be given to using a wide range of analytical techniques exploiting different physico-chemical properties of the molecule; for instance, size, charge, isoelectric point and hydrophobicity. A list of the analytical possibilities is beyond the scope of this guideline. In the following there are only examples of the type of analysis.

7.1.2 Structural evidence for the active substance (including comparison with reference or natural product)

Sufficient sequence information to characterise the gene product adequately should be obtained. The degree of sequence verification required will depend on the size and complexity of the molecule, considering the extent of other characterisation tests. In most instances, determination of the entire sequence can be obtained after HPLC separation and sequencing of the peptides released by enzymatic digestion. Attention should be paid to the possible presence of N-terminal methionine and N-formyl methionine, signal or leader sequences, other possible N- and C-terminal modifications (proteolytical processing). It should be considered integrating modern mass spectrometry techniques in characterising the primary structure.

7.1.3 Post-translational modifications

Apart from the proteolytical processing, potential types of post-translational modifications are N- and O- glycosylation and for instance acetylation, hydroxylation and gamma-carboxylation. In addition, there are post-translational modifications that occur as degradation products such as deamidation and oxidation.

Some rDNA products are glycoproteins. There is a large range of oligosaccharide structures and these substances are characterised by glycoform heterogeneity both in their natural forms and those resulting from rDNA technology. The detail of this heterogeneity can be affected by many factors and the glycosylation pattern may have an important role in determining activity, particularly in vivo. The extent of analysis undertaken should depend on the role played by the carbohydrate moiety where this is known. A range of different analytical techniques should be explored, for instance quantitative isoelectric focusing or capillary electrophoresis, anion exchange chromatography for monosaccharide component analysis and oligosaccharide determination, lectin affinity chromatography, mass spectrometry.

7.1.4 Conformational data for macromolecules

It is desirable to include suitable tests to establish that the product has the desired conformational structure and state of aggregation. Examples of techniques suitable for such purposes are: polyacrylamide gel electrophoresis; isoelectric focusing; size exclusion, reversed phase ion exchange, hydrophobic interaction or affinity chromatography; peptide mapping and subsequent amino acid sequencing; light scattering; UV spectroscopy; circular dichroism and mass spectrometry. Additional characterisation of the product using for example NMR spectra, X-ray crystallography or relevant immunochemical techniques may provide valuable information.

7.1.5 Biological, immunological characterisation, expression of strength

Biological and immunological characterisation should include as wide a range of techniques as necessary. The specific activity of highly purified material should be determined (units of activity/weight of product).

When appropriate the biological activity of the product and its physical characteristics, including the amino acid sequence, should be compared with that of a highly purified preparation of the naturally occurring molecule.

7.2 Purity

Data should be provided on contaminants whose presence is anticipated in the final processed product. The level of contamination considered as acceptable should be justified, and criteria for acceptance or rejection of a production batch should be given. It is important that the techniques used to demonstrate purity be assessed using as wide a range of methods as possible, including physico-chemical and immunological techniques. Unwanted materials of host origin, as well as materials which may have been added during the production or purification processes, and where appropriate, viral and nucleic acid contamination should be tested.

8. CONSISTENCY AND ROUTINE BATCH CONTROL OF BULK FINAL ACTIVE SUBSTANCE

A comprehensive analysis of the initial batches of a product should be undertaken to establish consistency with regard to identity, purity and potency. Thereafter, a more limited series of tests may be appropriate as outlined below. A clear difference should be made between the analytical tests performed during the development, in order to fully characterise the active substance and tests performed routinely on each production batch of purified bulk product.

8.1. Consistency

An acceptable number, for example 5 (smaller numbers could be acceptable where justified), of successive batches of the bulk processed product should be characterised as fully as possible to determine consistency of composition. In the case of a production where multiple harvests are applied, batches from different fermentation runs should normally be studied. The studies should include biological, chemical and immunological methods to characterise and assay the active substance (including methods showing the consistency of the glycosylation pattern for glycoproteins) and methods to detect and identify impurities. Any differences which occur between batches should be noted.

8.2. Routine batch control analysis

8.2.1 Identity

A selection of the tests used to characterise the purified active substance (see 7.1) should be used to confirm the product identity for each batch. The methods employed should include tests for the physico-chemical and immunological characteristics, together with test for the anticipated biological activity. Depending on the extent of other identification tests, sequence verification of a number of amino acids at the N- or C-terminus or other methods such as peptide mapping should be performed.

8.2.2. Purity

The degree of purity desirable and attainable will depend on several factors; these include the nature and intended use of the product, the method of its production and purification and also the degree of consistency of the production process. In general, a very high degree of purity can be achieved for most products by modern manufacturing procedures.

The purity of each batch should be established and be within specified limits. The analysis should include sensitive and reliable assays for DNA of host cell origin and/or of the vector applied to each batch of product prepared from cell lines of mammalian origin, in which case upper limits should be set. It is recommended that DNA analyses are also performed on each batch of bulk product obtained from other eukaryotic cell systems and limits set for DNA content. DNA of prokaryotic expression systems should be tested for wherever appropriate to consideration of the quality of the product. The residual cellular proteins should also be determined by an assay with appropriate sensitivity (e.g. ppm) and strict upper limits set. In some instances, potential impurities such as DNA can only be determined on intermediates of purification, at an earlier step.

8.2.3. Test for potency

The potency of each batch of the product should be established (e.g. units of biological activity per ml) using, wherever possible, an appropriate national or international reference preparation calibrated in units of biological activity (see section 9).

In addition, information on specific activity (units of biological activity per unit weight of product) will be of considerable value and should be reported. A highly purified reference preparation is required to standardise measurements of specific activity (see section 9).

It is recommended that correlation between potency measurements, involving biological tests, and the results of physico-chemical methods of assay are made and the information reported. If possible, batches should be calibrated using accurate physico-chemical tests, and the biological assays used to confirm -within stated limits- that the product is biologically potent.

9. SPECIFICATION AND REFERENCE MATERIALS

The studies described in section 7 will contribute to a definitive specification for the product when justified by the information obtained from the examination of successive batches and results of batch analysis, as indicated in section 8.

A suitable batch of the product, preferably one which has been clinically evaluated, should be fully characterised in terms of its chemical composition, purity, potency and biological activity, including where possible full amino acid sequencing, and retained for use as a chemical and biological reference material.

Criteria for expiration and possible re-testing and re-qualification of reference standards should be established.

10. FINISHED PRODUCT AND DEVELOPMENT PHARMACEUTICS

The development of the formulation should be described in detail and justified, particularly with regard to the presence and amount of stabiliser such as albumin and/or detergents. The product in final containers should be shown to comply with the requirements of the European directives and pharmacopoeias. In circumstances where this is not possible the omission of tests should be justified by the manufacturer.

GLOSSARY

1. Cell Banks

a) Master cell bank (MCB)

A homogeneous suspension of the original cells already transformed by the expression vector containing the desired gene, aliquoted into individual containers for storage (e.g. in a liquid nitrogen refrigerator). In some cases it may be necessary to establish separate master cell banks for the expression vector and the host cells.

b) Working cell bank (WCB)

A homogeneous suspension of cells derived from the master cell bank(s) by a finite passage level, aliquoted into individual containers for storage (e.g. in a liquid nitrogen refrigerator).

In both cell banks, all containers are treated identically during storage, and once removed from storage, the containers are not returned to the cell bank stock.

2. Production method

a) Production at finite passage (single harvest)

This cultivation method is defined by a limited number of passages or population doublings which must not be exceeded during production.

b) Continuous culture production (multiple harvest)

The number of population doublings (or duration of culture for certain production systems) are specified based on information concerning the stability of the system and the consistency of the product. Criteria for the termination has to be defined by the manufacturer.

3. Bulk harvest

This is a homogeneous pool of individual harvests or lysates which is processed in a single purification run.

4. Bulk final active substance

This is the final product, after completion of the production process, obtained from a bulk harvest. It is maintained in a single container or multiple identical containers where necessary and used in the preparation of the final dosage form. The generation of this final batch has to be clearly defined and unambiguously recorded by the producer.

5. Finished product

The active substance is formulated and filled into final, sealed containers which hold the product in its final dosage form, i.e. the finished product. The containers of a filling lot are processed together and uniform in their contents and biological potency.

USE OF TRANSGENIC ANIMALS IN THE MANUFACTURE OF BIOLOGICAL MEDICINAL PRODUCTS FOR HUMAN USE

Guideline Title	Use Of Transgenic Animals In The Manufacture Of Biological Medicinal Products For Human Use
Legislative basis	Directive 75/318/EEC as amended
Date of first adoption	December 1994
Date of entry into force	July 1995
Status	Last revised December 1994
Previous titles/other references	none/III/3612/93
Additional Notes	This document is concerned with the use of transgenic animals to produce biological pharmaceutical materials for use in human recipients.

CONTENTS

USE OF TRANSGENIC ANIMALS IN THE MANUFACTURE OF BIOLOGICAL MEDICINAL PRODUCTS FOR HUMAN USE

1. INTRODUCTION

Transgenic organisms contain a foreign gene which has been experimentally inserted into the normal genetic component, and currently include many plants and a number of animal species. They have been used experimentally to investigate gene function, development and disease. Transgenic animals have also been proposed as a means of testing agents for oncogenicity or virulence.

This document is concerned with the use of transgenic animals to produce biological pharmaceutical materials for use in human recipients. Transgenic animals may produce higher quantities of material in more concentrated form than existing culture methods, and therefore have considerable advantages in both the cost of producing the starting material and in its downstream processing. In some instances where very large amounts of material are required for therapy the use of transgenic animals may be one of the few viable production strategies. However in some respects the products resemble classical biologicals in that they derive from a whole animal rather than from definable culture systems. The considerations which apply are therefore a blend of those relevant to recombinant DNA (rDNA) derived materials and materials from less defined sources.

2. DEFINITIONS

Forebears: the animals from which the egg and sperm used to create the genetic founder were derived

Host: the recipient mother in whom the embryonic genetic founder was implanted

Genetic founder: the transgenic animal resulting from the introduction of the foreign DNA into the embryo or fertilised egg

Production founder: a transgenic animal used as a source for the generation of production animal herds

Production animals: the immediate offspring of the production founder

3. SCOPE OF THE NOTE FOR GUIDANCE

Many different species have been considered or developed for the production of biological medicinal products and by use of appropriate targeting sequences the transgene has been expressed in body fluids such as blood or in milk as well as in other source tissues. A wide range of host animals and source materials are therefore possible each raising specific concerns. All products must be considered on a case by case basis. However the strategy adopted should be such as to minimise potential microbiological contamination during the creation of the transgenic line including potential contamination from the host and founder

animals. Maintenance of the production animals should be such as to minimise contamination of the starting materials such as milk or blood from which the final product will be purified. The purity and microbiological safety of the final product is of major concern.

The production facilities used will probably employ agricultural animals and techniques. It is important to bear in mind that the requirements for manufacture of pharmaceutical products will be more stringent than those for agricultural production, and the production process should be designed accordingly. This document emphasises products derived from fluids of transgenic animals, particularly milk, as there is at present considerable interest in such sources, but many of the considerations will also apply to other source tissues. Other relevant notes for guidance should be taken into account including those concerned with the validation of virus removal and inactivation procedures, minimising the risk of transmission of agents causing spongiform encephalopathy via medicinal products, the production and quality control of medicinal products derived by rDNA technology and the Biotech headings for Notice to Applicants (Part II of application file).

The veterinary and environmental issues relevant to animal welfare and the consequences of release have been considered elsewhere, (see for example Directives 90/219/EEC on contained use and 90/220/EEC on deliberate release of genetically modified organisms) and the animals used in production must comply with existing regulations concerning the development of transgenic animals.

4. THE TRANSGENIC ANIMAL

4.1 Origin

Animals which have been proposed as hosts for production include among others sheep, cows, pigs, rabbits and mice, and much interest currently centres on the use of transgenes expressed in milk or colostrum. The choice of animal will be determined by a variety of factors. For example pigs breed rapidly and produce large litters, so that establishing a suitable transgenic line of animals may be technically simpler than if the same process is attempted in cows. On the other hand pigs are difficult to milk, while milk production in cows is well understood.

Each species will raise its own microbiological and virological concerns which should be addressed. Many of the potential host animals are not conventional laboratory animals, but infectious agents of agricultural significance are likely to be well known. The microbiological status of the production animal, its forebears and host animals involved in derivation of the transgenic line should be documented as far as is possible. Consideration should be given to the use of breeds of animals resistant to specific agents such as scrapie resistant breeds of sheep. The founder animals and their offspring should be shown to comply with the existing guidelines *Minimising the Risk of Transmitting Agents causing Spongiform Encephalopathy via Medicinal Products.*

4.2 The expression system

The isolation and characterisation of the gene and associated control elements should be described as should the process by which the final construct was made. The strategy used to develop the particular expression system should be described and justified. In particular the rationale for the use of regulatory sequences to ensure correct expression of the gene in the

appropriate tissue should be clearly described. The complete sequence of the final construct should be determined.

4.3 Creation of the transgenic animal

A number of methods are currently in use for the creation of transgenic animals. One favoured method involves the inoculation of the DNA into the pronucleus of a fertilised ovum, followed by implantation into pseudo pregnant females. This results in a proportion of animals carrying the transgene in the germ line which may be high in some species (e.g. mouse 5-30%) or low in others (e.g. cows and sheep, 1-5%). Depending on the time when the transgene is incorporated into the cellular DNA, mosaic animals may develop in which certain cell lineages carry the transgene while others do not. Other methods of creating transgenic animals involving retroviral infection of the embryo at an early cleavage stage in the blastocyst result in only a proportion of the cells carrying the transgene, and therefore a high proportion of mosaic animals some of which may not have the transgene incorporated in the germ line at all. Methods for predictable site specific integration of sequences into the host genome would have advantages for both controlled expression and safety.

The method used to create the transgenic animal should be described in detail, including the isolation of ova, in vitro fertilisation, insertion of the transgene, reimplantation and delivery. The use of retroviral vectors raises additional quality considerations related to preparation of the vector, its virological purity and its persistence. Consideration of guidelines related to regulatory aspects of gene therapy is advisable.

The genealogy of the production animals must be documented. A transgenic line will derive from a single genetic founder animal, and materials from different transgenic lines should not be mixed. The founder animal and the production animals should be defined as diploid or haploid with respect to the inserted sequence.

The level of expression of the incorporated gene should be assessed and the tissue distribution of expression should wherever possible be shown to be consistent with the chosen strategy of expression. Estimates of the copy number should be made and evidence as to the accuracy of the incorporated gene sequence should be presented. It is believed that while multiple copies of the transgene are usually incorporated, there is usually only a single site of integration. Thus, even where multiple copies are introduced it will be possible to identify the expressed sequence or sequences with confidence at the level of the genomic DNA.

It is of doubtful value to determine multiple sequences of the insert but evidence that the correct sequence is present should be obtained. Some sequence data for example of cDNA clones will be valuable as will restriction endonuclease maps, which will serve to demonstrate that the site of integration has not changed in offspring of the founder animal where these are used. It should be clearly stated whether the animals used for production are haploid or diploid for the transgene. The animals used in production should be characterised to ensure an acceptable level of consistency.

The virological status of the donors and host animals should be shown to be acceptable; for example calves born to mothers infected with BVDV are likely to be persistently infected, and vertical transmission of BSE has not been eliminated as a possibility. Similarly bovine immunodeficiency virus (BIV) may be transmissible through semen. These are examples only.

4.4 Stability of the gene

Transgenic animals produced by microinjection of DNA have the highest probability of incorporating the transgene into the germ line and therefore expressing it in the appropriate intended tissue. However this method often results in the insertion of multiple head to tail copies of the transgene, and rearrangements and eliminations may occur on breeding. The stability of the gene on breeding will be an issue where numbers of animals derived from a founder animal are to be used. Greater consistency of production will be achievable if a uniform production herd can be bred in a reproducible manner. The strategy used to generate a herd of animals of similar productivity should be clearly delineated. Evidence should be presented that the animals are similar, in the yield of product and genetically in terms of numbers of copies of the gene incorporated and the site of integration in the genome. Restriction length polymorphisms may be of value in providing evidence for a constant integration site.

5. PRODUCTION

5.1 Housing and animal care

There are major veterinary and ethical difficulties in raising and maintaining agricultural animals under specific pathogen free conditions although this is desirable if it can be achieved. Otherwise good husbandry and agricultural practice may contribute to virological and microbiological safety. However the general conditions suitable for satisfactory agricultural production are likely to be less stringent than those applicable to the manufacture of pharmaceutical materials, so that good husbandry and agricultural practice are unlikely to be sufficient alone to ensure adequate safety of a pharmaceutical product. The conditions under which the animals are bred and maintained should be described and precautions taken to ensure that the site is free of disease likely to affect the production animal species prior to use. Potential sources of infection may include foodstuff, animal handlers and veterinary surgeons, and the environment especially if the animals are kept outside. The health and virological status of the animals should be documented and animals subjected to regular veterinary examination. If the source material is milk the health of the udder should be subject to special examination. Administration of antibiotics and hormones for prophylactic or therapeutic reasons at any time when they may contaminate the product is not permitted. Cows should be shown to be free of bovine tuberculosis.

Many cow herds are known to be infected with bovine viral diarrhoea virus, and other infections include bovine polyoma and infectious rhinotracheitis virus which may or may not be apparent. Sheep are susceptible to many agents including orf virus and Louping Ill virus, and pigs to swine vesicular disease and porcine parvovirus. These examples do not constitute an exhaustive list. Many infectious agents of agricultural animals may establish persistent infections, and some are also able to infect humans. In general animals which are known to be infected with an agent should not be used for production.

5.2 The source material

Different litter mates have been reported to express the transgene to different levels for unknown reasons unrelated to copy number or accuracy of the incorporated sequence. During the period of lactation the expression of the gene may vary, and it may also vary between different lactations. The source material may therefore be variable, making

purification procedures potentially less consistent. The nature of the source material (for example milk or colostrum) should be clearly stated and justified. There is wide variation in the composition of milk and the purification process must be shown to be satisfactory in dealing with the range of materials expected. Acceptable limits for the level of active substance in the source material should be set. Where the source material is milk, specifications could be set in terms of product activity per unit of non fat dry solid. A single batch of source material may involve pooling separate harvests and should be clearly defined. While milk is a source material with a long history in which the safety issues are generally well understood, pharmaceutical proteins may be given parenterally, not orally, and it may not be possible to pasteurise or sterilise them in the ways which have been applied to milk.

Limits for the microbiological status of the source material should be set. Milk is likely to be contaminated with bacteria, although such contamination may be minimised by good husbandry. Contamination by certain agents, such as zoonotic mycobacteria, would make the material unacceptable. While bacteria may be removed by sterile filtration of the product, mycoplasma may not and efforts should be made to exclude them from the source material.

5.3 Purity of the active substance and validation of downstream processing

The purity of the active substance should be in accordance with criteria accepted for products of rDNA technology. Most such products are currently manufactured by in vitro culture methods involving either the fermentation of microorganisms or the large scale culture of cells from higher organisms. A transgenic animal is unlikely to be free of pathogens to the same degree as a well characterised cell bank. Validation of the purification process is therefore important in ensuring the safety of the product. Guidelines on *Virus Validation Studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses* have been prepared. Where the source material is milk or colostrum, contamination with mycoplasma is possible, and the process should be validated for their removal, as well as limits set for their levels in the starting material.

The source material, whether blood, milk, colostrum or other tissue will contain large numbers of host derived proteins other than the desired product, some of which may be present in large amounts which must be removed. Milk is known to contain proteases, and the possible effect of these on the product should be addressed; if degradation occurs, acceptable limits should be set for the products in the final material. Care should be taken to document and if necessary eliminate host proteins homologous to the required product. Limits should be set for contaminants which may copurify with the desired material. Hypersensitivity to milk is common, and materials must therefore be of high purity.

Data on the carbohydrate components of the product should be presented. The non enzymic glycosylation or glycation of proteins in the presence of free carbohydrate such as lactose should be considered. This process is likely to be inevitable to some degree for a product derived from milk but attempts should be made to reduce it to a minimum. Glycated proteins can cause the activation of end stage macrophages to produce cytokines, and long term exposure to a glycated product is likely to be harmful.

The attractions of transgenic animals as a means of production include the ability to produce materials required on a scale which may otherwise be prohibitive because of the large amounts required in therapy. This increases the concerns associated with the immunogenicity of the proteins because of trace impurities or imperfect post translational

modifications, and close attention should be given to the purity, quality and consistency of the product.

6. CONCLUSION

Transgenic animals may have advantages over existing production methods with respect to the quantity and quality of the source material, which may reduce production costs and simplify downstream processing. Other than veterinary and environmental concerns, which are outside the scope of this document, the issues they raise are principally those of using a whole organism in production rather than a potentially more predictable cell culture or fermentation system based on a seed lot. These include microbiological and virological concerns, possible difficulties in purification and the consistency of the production and purification process.

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aeres

agence d'évaluation de la recherche
et de l'enseignement supérieur

Section des Unités de recherche

Evaluation report

Research Unit :

Physiopathogenesis and treatment of fulminant hepatitis and liver cancer

University Paris 11

December 2008

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agence d'évaluation de la recherche
et de l'enseignement supérieur

Section des Unités de recherche

Evaluation report

Research unit

Physiopathogenesis and treatment of fulminant hepatitis and liver cancer

University Paris 11

Le Président de l'AERES	Section des unités de recherche
	Le Directeur
Jean-François Dhainaut	**Pierre Glorieux**

December 2008

Evaluation report

The research unit :

Name of the research unit : "Physiopathogenesis and treatment of fulminant hepatitis and liver cancer"

Requested label : UMR_S

N° in case of renewal : U785

Head of the research unit : M. Didier SAMUEL

University or school :

University Paris 11

Other institutions and research organization:

INSERM

Date of the visit :

November 13th of 2008

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Members of the visiting committee

Chairman of the commitee :

M. Francesco NEGRO,Geneva

Other committee members :

Ms. Marina BERENGUER, Valencia, Spain

M. Steven DOOLEY, Heidelberg, Germany

M. Markus HEIM, Basel, Switzerland

M. Philippe MERLE, Lyon, France

M. Frederik NEVENS, Leuven, Belgium

CNU, CoNRS, CSS INSERM, représentant INRA, INRIA, IRD... representatives :

M. Fabien ZOULIM, CNU representative

M. Louis BUSCAIL, CSS INSERM representative

Observers

AERES scientific representative :

M. Pierre BEDOSSA

University or school representative :

M. Laurent BECQUEMIN, University

Research organization representative :

Mme Marie-Josephe LEROY, INSERM

Evaluation report

1 • Short presentation of the research unit

- Numbers of lab members :
  - Researchers with teaching duties : 11
  - Full time researchers : 3
  - PhD students : 9
  - Engineers, technicians and administrative assistant : 12
- Numbers of HDR : 8
- Numbers of PhD students who have obtained their PhD : 2 in 2 years (the unit was created in 2006)
- Average length of a PhD during the past 2 years : 2.5 years
- Numbers of PhD students currently present in the research unit : 9 including 8 with a fellowship
- Numbers of lab members who have been granted a PEDR : 0
- Numbers of "publishing" lab members : 14 out of 14 among researchers with teaching duties and full time researchers.

2 • Preparation and execution of the visit

During the visit, the committee had the opportunity to discuss with the Director, the Co-Director, the researchers, the students as well as the administrative and technical personnel, and review the scientific activity of the Unit spanning the period January 2006 to present. The Director, the Co-Director and some selected investigators presented their academic achievements with the help of slides, while the remaining personnel held more informal talks with the experts. Some administrative and budgetary information was also provided, although the fine details of the complete financial structure and support of the Unit remained vague and could not be appreciated in full.

3 • Overall appreciation of the activity of the research unit, of its links with local, national and international partners

The Unit 785 was formed at the end of 2005 by joining two former research groups, one more clinical research-oriented, the other essentially dedicated to basic studies, but both strongly committed to the study of various aspects of liver diseases, from the pathogenesis to the clinical management. The two leaders of the former groups had a long-standing experience in the research on various aspects of fulminant liver failure, viral hepatitis, and hepatocellular carcinoma, as shown by their extensive publications track record, and were appointed respectively Director and Co-Director. The major goal of this joint venture was to foster translational research exploiting the respective competences of the two research groups, while maintaining at the same time their specificities. Thus, the goals of this evaluation were to assess the scientific productivity during the last two years together with the degree of integration and synergism between the two former teams.

The research objectives of the Unit are focusing on (i) the treatment of acute liver failure, hepatocellular carcinoma and cholangiocarcinoma, (ii) the pathobiology of hepatitis virus infection in the setting of liver transplantation, with particular emphasis on the mechanisms of viral persistence, viral compartmentalization, the factors of liver disease progression including the mechanisms of virus-induced fibrogenesis, and (iii) the auto- and alloimmunity in the setting of liver transplantation.

From the clinical standpoint, the patients' recruitment rate of the Centre Hépatobiliaire, where the Unit is located, is impressive: in 2007, as many as 138 liver transplantations have been performed (approximately 15% are due to fulminant liver failure) and 300 new cases of primary hepatocellular carcinoma cases were seen. Clinical data and material are available for retrospective analyses, and the prospective enrolment of patients in clinical trials is assured.

The dual specificity of the Unit has attracted interactions with other institutions, both national (INSERM and CNRS research groups operating in the André-Lwoff Federative Research Institute at Paul-Brousse Hospital) and international (University La Sapienza, Rome, and the Universities of Dundee, Zurich and Aachen).

4 • Appreciation of resources and of the life of the research unit

The academic personnel consists of 6 university professors, 5 *maîtres de conférence* and 3 senior scientists (*chargés de recherche*): the latter ones have been recruited since 2006, i.e. after the unit was created. There are 8 additional investigators and 12 employees involved in the administration and technical support. Currently, there are 9 PhD students (two are MDs) and three Master students, recently recruited. Three PhD theses have been completed since 2006, and more are underway.

The investigators of the Unit have raised funding from several institutions and agencies (ANR, PNRHGE, INCA, ARECA, ANRS and the European Commission) for a total of 451K euros in 2006, 619K euros in 2007, and 734K euros in 2008. These figures include the annual support from INSERM and from the University Paris-XI. These funds have been obtained, sometimes repeatedly, by no more than 6 academic members of the unit.

The Unit has published several original papers in peer-reviewed journals: 5 in the second half of 2006, 10 in 2007 and 8 so far in 2008. These figures include both clinical and basic papers, and do not take into account editorials and review articles even when published in peer-reviewed journals. Half of the papers are collaborations: thus, the investigators of the Unit have signed, as last authors, 11/22 papers over the 3 years, contributing to 86.115 points of impact factor (almost 60% of the total). Five academic members of the unit have signed an original article as last (*senior*) author.

The investigators of the Unit have filed two patents: one for the *HIP/PAP polypeptide composition for use in liver regeneration and for the prevention of liver failure* (deposited in 2004, i.e. before the Unit was created, and published in 2006), and the other for the *Nucleic acids for expressing a polynucleotide of interest in mammalian cancer cells* (deposited in 2007).

5 • Recommendations and advice

— Strong points :

- Both team leaders have an outstanding track record in clinical and basic research respectively.

  The patients' recruitment rate is a major asset, as this is one of the pillars of all translational research programs.

- The scientific level and background of most permanent researchers are rather strong, and in a few cases impressive and convincing; some topics are novel (like the proteomics mapping of cholangiocellular carcinoma, the radioiodine liver mass imaging, or the mechanisms of fibrogenesis via EMT) and some cutting-edge (like the study of liver cell polarization).

e

The fact that the amount of competitive funds is increasing over the years is a very positive aspect of the vitality of the Unit (at least of some of its members, see below, among the weak points).

The fact of having patents is another very important asset and a strong premise to attract industrial partners: as a matter of fact, the Unit is working in close interaction with a start-up (ALFACT Innovation) for preclinical and clinical development of molecules potentially active in the treatment of fulminant liver failure (HIP-PAP protein, ALF-5755) and hepatocellular carcinoma (Adenovirus-HIP-NIS).

The melting process involving basic and clinical projects is certainly a difficult one and probably slower than initially planned, also owed to the complexity and multiplicity of the research topics that are pursued; the interview with the Director and the permanent researchers gave however the impression that the process is ongoing, and that there is a sense of direction in the overall research activity; in addition, this process benefits of the active support of a whole network of surgeons, virologists and pathologists.

The ongoing applied research projects are likely to proceed towards the clinical experimentation: in one case (involving the use of HIP/PAP, for which the GMP process is completed) a multicenter, Phase I trial in healthy volunteers should start at around mid-2009, and the protocol is currently being finalised; in the other case (involving the use of gene therapy with Adenovirus-HIP-NIS, a somehow higher-risk project than the previous one), contacts are underway for additional toxicology studies in the animal model (with a company based in San Francisco, CA), and therefore, if everything goes as planned, the Phase I study may start in 2010.

The Unit has been able to actively recruit scientists and fellows from outside institutions, including international ones, suggesting a certain level of attractiveness; this is not only true for senior scientists but also for PhD students; it was quite instructive through the discussion with the latter ones, since it underscored the level of enthusiasm of these young fellows and their competitiveness (all students in their third year or higher had at least one paper published or in press); on the other hand, the meeting with the administrative and technical personnel underlined another aspect of the working environment, i.e. that of a deep respect of the professional expectations of all human resources (this detail was particularly appreciated by the committee).

— Weak points and recommendations :

- Although the clinical research studies are important and have impacted on the clinical management of patients with fulminant liver failure, hepatocellular carcinoma and viral hepatitis in the setting of liver transplantation, they are all retrospective, as there is an evident lack of prospective validation studies; given the patients' recruitment rate, it is a bit surprising that this kind of approach has not been pursued further; thus, we recommend to focus more on prospective validation studies in the clinical setting;
- There is some concern that, in some cases, junior scientists may not receive sufficient encouragement to build their own group. In addition, some of the academic members do not seem to apply for their own research grants or conduct their own independent research. The team leaders should do every effort to stimulate all researchers to apply for their own grants and improve their own national and international visibility, given the potential of the institution. One has to consider that, among the 22 "publishing" members of the Unit, at least 11 (6 professors and 5 "maîtres de conférence") may have the status of group leader, only 5 have published an original article as last (*senior*) author during the past three years.
- Although the Unit has been started in 2006, and its scientific output cannot be appreciated in full, the number of publications in high-ranking scientific journals seems insufficient, given the potential of the Unit; thus, the number of publications in high-rank journals can and must be increased, involving the members of the unit who have not sufficiently contributed so far;
- Some experts raised the concern that in a few cases the experimental design of the scientific projects may not be sufficiently systematic and mechanism-oriented: to consolidate the current achievements, lab meetings and progress reports, with outside experts, should be implemented with higher frequency;
- There was almost a consensus that some research topics seem less attractive and may not lead to major breakthroughs, such as that on the autoimmunity in the setting of liver transplantation.

The committee wondered whether some degree of reorganization may even benefit the other, more attractive sectors of research.

Note de l'unité	Qualité scientifique et production	Rayonnement et attractivité, intégration dans l'environnement	Stratégie, gouvernance et vie du laboratoire	Appréciation du projet
A	A	A+	A	A

Appendix J

UNIVERSITÉ PARIS-SUD 11

Le Président de l'Université Paris-Sud 11

à

Monsieur Pierre GLORIEUX
Directeur de la section des unités de recherche
AERES
20, rue Vivienne
75002 Paris

Orsay, le 20 mars 2009.

N/Réf. : 72/09/GCo/LM/LS

Objet : Rapport d'évaluation d'unité de recherche
N° S2100012396

Monsieur le Directeur,

Vous m'avez transmis le vingt trois février dernier, le rapport d'évaluation de l'unité de recherche «Pathogénèse et traitement de l'hépatite fulminante et du cancer du foie» - UMR S 785 , et je vous en remercie.

L'université prend bonne note de l'appréciation et des suggestions faites par le Comité.

Les points à améliorer seront discutés avec le directeur d'unité dans un esprit constructif pour l'avenir de la recherche à l'université.

Vous trouverez en annexe les éléments de réponse de monsieur Didier SAMUEL, Directeur de l'unité de recherche.

Je vous prie d'agréer, Monsieur le Directeur, l'expression de ma sincère considération.

Guy COURRAZE
Président

UNIVERSITÉ PARIS-SUD 11 - Président - 91405 ORSAY CEDEX

P.S. : Les représentants de l'université Paris-Sud 11 étaient Mme Anita BERSELLINI – Présidente et Mr Laurent BECQUEMONT – Médecin Enseignant Chercheur.

P.J. : Commentaires de Mr SAMUEL

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Appendix J

Unité de recherche U785
Pathogenèse et traitement des Hépatites
Fulminantes et du Cancer du Foie

Inserm

Institut national
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Villejuif, 9 March 2009

Answer to the report from the AERES visiting committee of Research Unit UMR 785 : Physiopathogenesis and treatment of fulminant hepatitis and liver cancer, Inserm-University Paris 11

We thank the visiting committee for this report. We thank the committee for the strong points of the Unit underlined in the report.

Regarding the weak points and the recommendations, we have the following answers.

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a) We acknowledge the importance of developing prospective studies and we will develop much stronger active collaboration between clinical and research teams.

b) We are of course willing to encourage young junior scientists from the team to develop their own group and apply for their own research grants.

c) Regarding the publications, we acknowledge the fact that we are still lacking major publications in top rank general scientific journals, however we were able to produce seven papers in the highest ranking journals of the specialty with IF > 10 (Gastroenterology, Hepatology) within the last 3 years. Since the visit of the committee, we have 3 additional papers in favorable revision: 2 on HIV and Liver Transplantation in the Journal of Hepatology (IF 6.6) and AIDS (IF 5.8), 1 on autoimmune hepatitis post-transplantation in the American Journal of Transplantation (IF 6.4); 1 paper on the relation between EMT TGF beta and HCV core is now accepted for publication in Plos one (Battaglia S et al. Liver cancer-derived hepatitis C

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virus core proteins shift TGF-Beta responses from tumor suppression to epithelial-mesenchymal transition Plos One 2009 ; 4(2).

d) Lab meetings are already frequent (every week), in addition a monthly meeting of the "Virus group" joins together clinical and research teams. However, as suggested by the committee, we will encourage the development of Mechanisms-oriented lab meeting and Progress reports.

e) Regarding the last point about auto and allo immunity in liver transplantation. This research group is currently growing, and has published several papers within the past 3 years including one in Hepatology (IF 10.6) and one in Proteomics ; an additional one is currently under revision in the American Journal of Transplantation (IF 6.4). Considering the correlation between emergence of autoimmune hepatitis and transplantation, this project is fully integrated in the thematic of the Unit. This group is an example of the relationship between clinical interest and fundamental mechanistic insights that we wish to develop.

Yours sincerely

Prof Didier SAMUEL
Director Inserm-Paris XI Unit U785

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